Cell Biology of Rat Spermatozoa

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Contents

Chapter 2 General Materials and Methods

Chapter 3. The Impact of Epididymal Maturation on Tyrosine phosphorylation of Rat Spermatozoa

Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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For my Mam.

Abstract

Cell Biology of Rat Spermatozoa

The purpose of the research presented in this thesis was to investigate the cell biology of rat spermatozoa. An additional aim was to utilise the knowledge obtained to aid the development of *in vitro* functional tests for the assessment of rat sperm fertility and identify potential markers of normal epididymal maturation.

As mammalian spermatozoa migrate through the epididymis, they acquire the potential for fertilisation, characterised by the acquisition of the ability to express co-ordinated movement and the competence to undergo capacitation. The mechanisms by which epididymal maturation confers upon mammalian spermatozoa the potential to capacitate is poorly understood. These studies investigated the impact of epididymal maturation on the signal transduction pathways regulating tyrosine phosphorylation using the laboratory rat as an animal model, since this signal transduction pathway is thought to be central to the attainment of a capacitated state and expression of hyperactivated motility, both of which are prerequisites for fertilisation.

Western Blot and immunocytochemical analysis demonstrated that epididymal maturation is associated with a progressive loss in phosphotyrosine expression located to the acrosomal domain. These differences in phosphotyrosine expression between caput and caudal epididymal spermatozoa appeared to reflect the normal *in vivo* situation. In addition, epididymal maturation of rat spermatozoa is also associated with an acquired competence to respond to high levels of intracellular cAMP by phosphorylating tyrosine residues on the sperm tail.

Epididymal maturation also lead to unique differences in the generation of reactive oxygen species (ROS) by spermatozoa obtained from the caput and caudal regions of the epididymis. Spermatozoa from both regions of the epididymis spontaneously generated equal levels of $O_2^{\cdot-}$ whereas only mature caudal spermatozoa

generated significant levels of H_2O_2. In contrast, although both caput and caudal spermatozoa generated increased $O_2^{\cdot-}$ in response to NADPH, induced levels were significantly greater in the immature caput cells. The effect of NADPH on tyrosine phosphorylation was also dependent on the maturation status of the cells as although it increased intracellular cAMP levels in spermatozoa irrespective of the region from which they had been obtained, tyrosine phosphorylation of tail proteins was only observed in the mature caudal spermatozoa.

These studies also demonstrated the requirement for bicarbonate in the incubation media for the maintenance of $O_2^{\cdot-}$ generation and tyrosine phosphorylation. Calcium was found to be a primary regulator of $O_2^{\cdot-}$ generation and tyrosine phosphorylation in rat spermatozoa, as both these processes were significantly stimulated when calcium was removed from the incubation media. In addition, phosphorylation of tyrosine residues on caput sperm tails was induced under these calcium free conditions. A possible explanation for the enhanced inhibitory effects of calcium on phosphorylation of the tail in caput spermatozoa could be the presence of calcium dependent phosphatases.

As the acrosome reaction is generally referred to as the endpoint of capacitation, the established acrosome reaction test assay was adapted to assess the capacitation and fertility status of rat spermatozoa following various treatments. Progesterone, a physiological inducer of the acrosome reaction following capacitation in mammalian spermatozoa, was also found to induce the acrosome reaction in mature caudal rat spermatozoa following a period of incubation with stimulators of intracellular cAMP. The reproductive toxicant, alpha chlorohydrin was found to impair the ability of rat caudal spermatozoa to undergo the progesterone-induced acrosome reaction. Tyrosine phosphorylation in caudal spermatozoa was also significantly inhibited following incubation with this compound *in vitro*.

These findings provide us with valuable biochemical markers to further elucidate the mechanisms of epididymal maturation and also the processes by which spermatozoa acquire the potential for fertilisation as they transit the epididymis.

Abbreviations

α CH	α-chlorohydrin
AC	Adenylyl cyclase
AH	Arachis Hypogea
AKAP	PKA anchoring protein
AMPS	Ammonium persulphate
ANOVA	Analysis of variance
APAAP	Anti-alkaline phosphatase
APES	3-Aminopropyltriethoxysilane
AR	Acrosome reaction
ATP	Adenosine triphosphate
5'AMP	5'-Adenosine monophosphate
BWW	Biggers Whitten and Whittingham media
cAMP	Cyclic Adenosine monophosphate
CHAPS	3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate
COX	cyclooxygenase
Cyc A	Cyclosporin A
DAG	Diacylglycerol
dbcAMP	N6, 2-O-dibutyryladenosine 3:5-cyclic monophosphate
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EHD 1	Ethidium Homodimer 1
GABA	γ-Aminobutyric acid
HRP	Horseradish peroxidase
IP_3	Inositoltrisphosphate
LOX	Lipoxygenase
NADPH	β Nicotinamide adenine dinucleotide phosphate, reduced form
NADH	β Nicotinamide adenine dinucleotide, reduced form
OA	Okadaic Acid
P_4	Progesterone
PBS	Phosphate buffered saline
PDE	Phosphodiesterases
PIP_2	Phosphatidyl-inositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLA	Phospholipase A
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinases
PTP	Protein tyrosine phosphatases
PTX	Pentoxifylline
PY	Phosphorylation of tyrosine residues
RAM	Rabbit anti mouse immunoglobulins
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBS	Tris buffered saline
TEMED	N, N, N', N' Tetra-methylethylenediamine
TRIS	Trizma base
ZP	Zona pellucida
ZP1	Zona pellucida glycoprotein 1
ZP2	Zona pellucida glycoprotein 2
ZP3	Zona pellucida glycoprotein 3

Chapter One:

General Introduction and Literature Review

Chapter 1. General Introduction and Literature Review

1.1. Aims of project

Spermatozoa are highly specialised cells and the increasing demand for fertility treatment has brought to the attention of researchers, the need for investigations into the complex sperm cell biology involved in the acquisition of fertility, in order to maximise the success of such procedures. In contrast with other areas of research, whereby investigations in species other than humans predominate, there is a wealth of literature relating to the cell biology and functionality of human spermatozoa. However, in comparison with work in the human and many other species, a limited amount of investigation has been carried out on rat spermatozoa, with regards to the basic cell biology and mechanisms behind the acquisition of function by these sperm cells.

If we know so much about human spermatozoa, what is the relevance of focusing on investigations in the rat?

In recent years, questions have been raised relating to the use of animals to determine the effects of drugs on spermatozoa. In the pharmaceutical industry, the rat is used globally as a reproductive toxicological model. Traditionally, rats are dosed with potential drugs and subjected to histological analysis of their reproductive organs, to identify drug-related pathology. Mating studies are also carried out, whereby male rats are mated with females which are then examined to determine whether or not fertilisation has taken place. In addition, the offspring are examined for drug-related pathology. Such procedures for identifying drug-related reproductive pathologies and loss of function are useful in their own right, but are they adequate? Do they provide enough information relating to the mechanisms by which drugs actually affect male reproductive function? The simple answer is no. An article published in Toxicology, *in vitro* (Aitken, 1990), discussed the possible use of *in vitro* tests of human sperm function, as diagnostic tools for toxicological analyses. Indeed such tests have a potential use for toxicological analyses at a cellular level, but there is still a need for traditional style *in vivo* dosing studies. Ethically, it is not possible to administer a human male with an unknown drug in order to determine whether there are any effects on sperm function. It is first essential that thorough reproductive toxicity data be obtained using an animal model.

As desirable as it would be to replace animal testing with other means of analysis, it is unlikely that suitable alternatives will be universally available in the near future. As a consequence it is vital that any animal chosen for safety studies must be a suitable model for the human. It is unlikely that the rat will be superceded by another animal model for preliminary safety toxicology studies. This is mainly due to the fact that they are cheap, easily available and in regards to public image, using rats is far more acceptable than dogs, cats and primates.

If the rat is to be used as a reproductive toxicological model then this raises the question of how useful is it as a model for humans? The answer to this question is still unresolved. As mentioned previously, histological analyses are a useful tool for detecting gross drug-related testicular pathologies and severe reductions of fertility. However, when considering the process of spermatogenesis, the rat appears to have better control over this process than man. In the average human ejaculate it is perfectly normal to observe a relatively high percentage of morphologically abnormal spermatozoa, in addition to immotile or even degenerative sperm cells. In contrast to the fragile nature of human spermatozoa, it is extremely rare to see morphologically abnormal rat sperm. Indeed throughout my PhD studies I have only ever observed two deformed sperm cells. Rat spermatozoa are also thought to possess a higher threshold in terms of their responsiveness to toxic insult. Therefore if the reproductive function of a male rat is compromised by a drug then it is almost certain that human sperm would also be affected. This difference in responsiveness presents a problem in terms of establishing minimum toxicity levels in the rat that will be meaningful for the human. In order to address this problem there is an urgent need to develop more sensitive functional tests of reproductive competence in the rat, that would not only indicate whether the fertilising capacity of the spermatozoa has been compromised, but also provide insights into the underlying pathophysiological mechanisms.

By investigating the cellular mechanisms by which a rat spermatozoon acquires its capacity to engage in successful fertilisation, it should be possible to determine the extent to which this species reflects the human and can be used as a model to generate clinically relevant data in the field of reproductive toxicology. Such fundamental information should also enable the development of suitable *in vitro* assays to detect changes in the functional competence of sperm populations, providing information that could be extrapolated to expose potential risks of reproductive impairment in the human. Computer assisted semen analysis (CASA) has been successfully used to identify drug-related changes in motility. However, this addresses only one parameter of sperm function, requires expensive equipment and can be very time consuming. It has also proved difficult to develop standardised parameters for normal and abnormal

sperm motility. Consequently it is not entirely suitable as a toxicological tool for routine analysis. In addition, assays such as the acridine orange test have been used to assess the effect of reproductive toxicants such as 1, 3-dinitrobenzene on DNA integrity in hamster spermatozoa (Peiris and Moore, 1998). The Comet assay which also measures DNA damage in spermatozoa has also been used in the human (Hughes *et al.*, 1999; Irvine *et al.*, 2000).

The aims of this study were to investigate the cell biology of rat spermatozoa, with the underlying intent of providing information that may be used in the development of *in vitro* functional tests, for the assessment of rat sperm fertility. Such tests could provide an additional useful toxicological tool for investigations into drug-induced impairments of sperm function.

1.2. Spermatogenesis

The mature spermatozoon is an elaborate, highly specialised cell and the process by which it is made is referred to as spermatogenesis. Spermatogenesis takes place in the seminiferous tubules of the testis (Fig. 1.1.) and may be split up into three elements: mitotic proliferation, meiotic division and extensive cell modelling, also referred to as spermiogenesis. Large numbers of spermatozoa are produced, approximately 300-600 sperm cells per gram of testis per second (Johnson and Everitt, 1995).

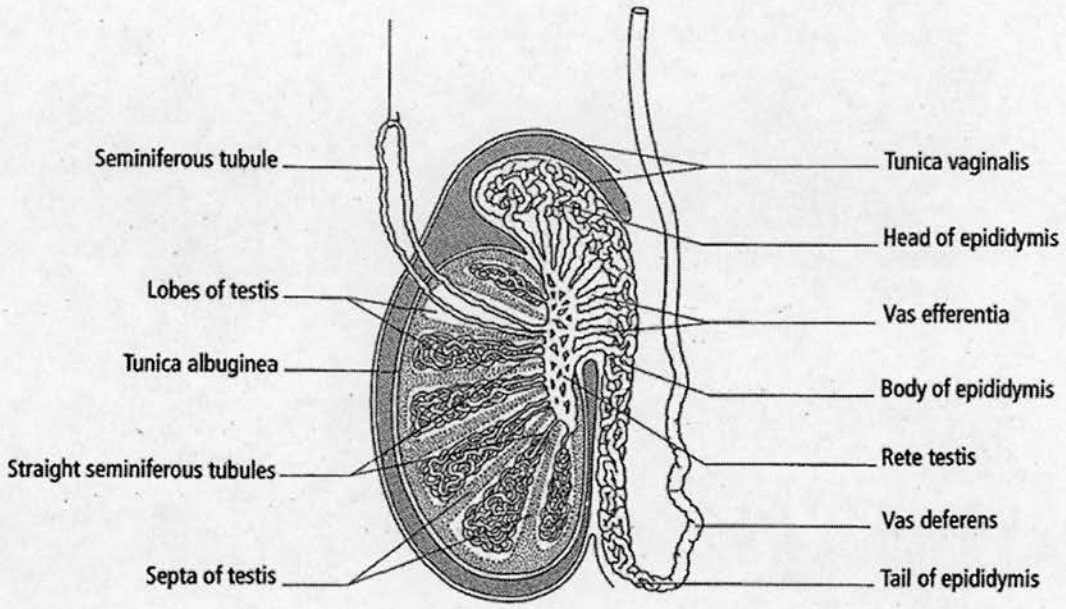


Figure 1.1. Transverse section through an adult human testis. Adapted from (Johnson and Everitt, 1995).

1.2.i. Mitotic proliferation

The emergence of A1 spermatogonia marks the beginning of spermatogenesis and each cell undergoes a limited number of mitotic divisions, thus producing a number of clones (Johnson and Everitt, 1995). The number of divisions varies from species to species and in the rat there are six divisions leading to a maximum clone size of 64 cells, although this number does not take into account the potential cell death associated with mitosis (Johnson and Everitt, 1995). The final mitotic division results in the production of resting primary spermatocytes. Interestingly, during mitotic division,

although nuclear division is completed successfully, cytoplasmic division is incomplete, resulting in cytoplasmic bridges linking all primary spermatocytes, derived from one type A1 spermatogonium. This syncytial organisation is only broken up during the last stages of spermatogenesis, known as spermiation, when the cells are released as single mature spermatozoa into the lumen of the seminiferous tubule.

1.2.ii. Meiosis

Primary spermatocytes commence the first meiotic division. There is a long prophase and the appearance and behaviour of the chromosomes during this phase enables classification of primary spermatocytes into five categories: Leptotene; during this phase single electron-dense threads appear (the condensing chromosomes). Zygotene; the threads pair and correspond to the bivalents formed by homologous chromosomes. Between the paired threads, a central linear electron-dense component develops, forming a tripartite structure, being termed a synaptinerial complex. Pachytene; the synaptinerial complex persists throughout this phase. Diplotene and Diakinesis; the synaptinerial complex disappears during these phases.

It is during the prolonged meiotic prophase, particularly during pachytene, the spermatocytes are especially susceptible to damage, resulting in potential widespread degeneration (Johnson and Everitt, 1995). This first division ends with the separation of homologous chromosomes to opposite ends of the cell on the meiotic spindle, after which cytoplasmic division yields, from each primary spermatocyte, two secondary spermatocytes, each containing a single set of chromosomes (Johnson and Everitt, 1995). Each chromosome consists of two chromatids joined at the centromere. They separate, move to opposite ends of the second meiotic spindle and the short-lived secondary spermatocytes divide to yield haploid early spermatids (Johnson and Everitt, 1995). Therefore, in the rat, from the maximum of 64 primary spermatocytes that entered meiosis, 256 early spermatids could result, although this is unlikely due to the high probability of sperm loss during this stage. A key factor in sperm output is the efficiency of spermatogenesis. Basically, how many germ cells can each Sertoli cell guide through development into spermatozoa? This varies from species to species, with the rat being near the top of the scale and man being placed firmly at the bottom of the league with the most inefficient spermatogenesis (Sharpe, 1994) (refer to Table 1.1.).

At the end of the meiotic phase, the important chromosomal reduction events of spermatogenesis are completed (Johnson and Everitt, 1995).

Species	Nº of elongate spermatids per Sertoli cell	Daily sperm production per gram of testis (10^6/g)
Rabbit	12.2 ± 2.0	25
Hamster	10.8 ± 1.4	24
Rat	10.3 ± 1.6	24
Rhesus monkey	-	23
Boar	-	23
Ram	-	21
Stallion	11.5 ± 1.0	16
Bull	-	13
Cynomolgous monkey	8.2 ± 3.6	-
Orang-utang	5.7 ± 2.3	-
Human	3.9 ± 0.5	4.4

Table 1.1. Comparative efficiency of spermatogenesis in a number of mammals using either the elongate spermatid, or the rate of daily sperm production per gram of testis. Adapted from (Sharpe, 1994).

1.2.iii Spermiogenesis and Spermiation

The transformation of spherical spermatids into the characteristic 'tadpole'-shaped spermatozoa is referred to as spermiogenesis. During this process a tail is generated for forward propulsion; the midpiece forms, containing the mitochondria which generate energy for the cell; the equatorial and postacrosomal cap region forms which is vital for sperm-oocyte fusion; the acrosome develops for penetration of the oocyte investments and the nucleus contains the compact packaged haploid chromosomes. The residual body acts as a dustbin for superfluous cytoplasm and is phagocytosed by the Sertoli cell following the departure of the spermatozoon (Johnson and Everitt, 1995). Spermiogenesis is completed with the formation of a fully mature spermatozoon that consists of a head and a flagellum. The head consists of the acrosome, nucleus, cytoskeletal elements and cytoplasm. The flagellum consists of an axoneme surrounded by outer dense fibres, the anterior portion of which has mitochondria wrapped around the outer dense fibres in a helical configuration. The posterior portion of the flagellum has a fibrous sheath surrounding the outer dense

fibres, both of which make up the cytoskeletal elements of the sperm flagellum (Eddy and O'Brien, 1994). The flagellum, like the head, is closely wrapped by the plasma membrane and contains little cytoplasm (Eddy and O'Brien, 1994).

A unique feature of the spermatozoon is that the plasma membrane is a mosaic of restricted domains, reflecting the specialised functions of surface and cytoplasmic components of the spermatozoon (Eddy and O'Brien, 1994) and references therein). The plasma membrane of the mammalian sperm head can be split into the acrosomal region and the postacrosomal region (Eddy and O'Brien, 1994) (Fig. 1.2.). The plasma membrane of the acrosomal region may be subdivided further into three domains: (1) the marginal segment which consists of the apical segment, anterior band and peripheral rim over the anterior margin of the acrosome, (2) the principal segment over the major portion of the acrosome and (3) the equatorial segment over the posterior part of the acrosome (Eddy and O'Brien, 1994) (Fig. 1.2.). Together, the marginal and principal segments are sometimes referred to as the anterior acrosome or acrosomal cap. The postacrosomal region includes the plasma membrane between the posterior margin of the acrosome and the neck (Eddy and O'Brien, 1994) (Fig. 1.2.).

The posterior ring is at the junction between head and tail and apparently forms a tight seal between the cytoplasmic compartments of the head and tail of the spermatozoon (Eddy and O'Brien, 1994). The plasma membrane of the flagellum is divided into the middle piece overlying the mitochondrial sheath and the posterior piece, which consists of the principal piece and distal tail (Eddy and O'Brien, 1994). The tail segments are separated by a fibrous cytoskeletal ring referred to as the annulus, which appears to adhere to the cytoplasmic surface of the plasma membrane (Eddy and O'Brien, 1994).

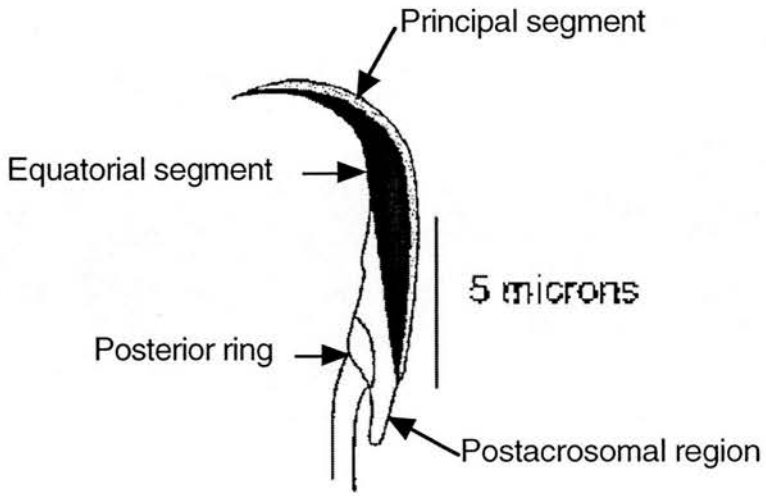


Figure 1.2. Diagram of a rat sperm head, illustrating the subdivisions of the plasma membrane overlying the rat sperm head. Adapted from Yanagimachi (Yanagimachi, 1994).

On completion of spermiogenesis, the thin cytoplasmic bridges that make up the syncytium rupture and the cells are released into the lumen of the seminiferous tubule in a process called spermiation (Johnson and Everitt, 1995). Spermatozoa are released into the lumen in a fluid secreted by the Sertoli cells so that a continuous flow, rich in spermatozoa washes towards the rete testis (Fig. 1.1.). As the fluid passes through the rete testis, the composition of its ions and small molecules changes, probably mainly by diffusional equilibration through the tubule walls, as the absence of inter-Sertoli cell junctions renders the blood-testis barrier much less complete (Johnson and Everitt, 1995). The spermatozoa are then carried through the efferent ducts into the epididymis. If the efferent ducts are ligated, then fluid outlet is blocked and the seminiferous tubules literally 'explode' due to the accumulating fluid (Johnson and Everitt, 1995).

1.3. The Epididymis: Epididymal Maturation

The epididymis is a highly convoluted tubule, closely applied to the posterior surface of the testis, extending from the upper to lower pole of the testis and held firmly to the tunica albuginea by connective tissue. It is divided into three sections: the caput, corpus and cauda (Fig. 1.1. and 1.3.). The main functions of the caput and corpus epididymis are fluid resorption and sperm maturation, whereas the caudal region is mainly responsible for storage prior to ejaculation.

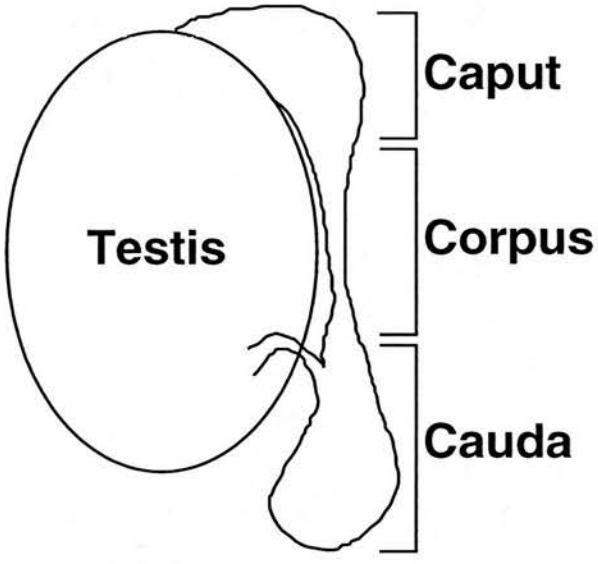


Figure 1.3. Schematic diagram of the different regions of the mammalian epididymis. Although there is some inter-species variation in organ shape, they all consist of the three regions referred to as the caput, corpus and cauda epididymis. The three regions may also be divided further into independent sections (refer to Fig. 2.1.).

Within the epididymis, most of the fluid in which the spermatozoa were transported from the seminiferous tubules of the testis, is reabsorbed, concentrating the spermatozoa 100-fold. In addition, the epididymis adds secretory products including carnitine, glycerophosphorylcholine, fructose and glycoproteins, the latter coating the surface of the spermatozoa (Johnson and Everitt, 1995). Passage through the efferent ducts and epididymis takes approximately 6-12 days in most species and it is during this migration through the epididymis, that they acquire the potential for fertilisation, characterised by the acquisition of (a) the potential to express co-ordinated movement and (b) the competence to undergo capacitation. These maturational changes in

functional capability are accompanied by both biochemical and morphological changes in the spermatozoa, some of which are summarised in Table 1.2.

Property	Details of changes
Concentration	100-fold; 50 x 10^6/ml entering suspended in fluid; dense packed at 50 x 10^8/ml on leaving
Completion of sperm modelling	Nuclear condensation and acrosomal shaping completed; cytoplasmic drop 'squeezed' down tail and shed
Metabolism	Cholesterol and phospholipids selectively metabolised, shifting lipid balance towards diacylglycerol, unsaturated fatty acids and desmosterol Increased dependence on external fructose for glycolytic energy production; little oxidative metabolism pH rises
Mobility	Increase in disulphide linkages between proteins in outer dense fibres of tail, yielding a more rigid flagellum with a stronger potential beat cAMP content of tail rises Acquires capacity for forward motion
Membrane	Coated with glycoproteins Rise in surface charge (due to sialic acid increase) and change in profile of surface proteins Membrane fluidity changes, as does its lipid composition

Table 1.2. Changes that occur in spermatozoa during the process of epididymal maturation. Adapted from (Johnson and Everitt, 1995).

The need for the epididymal maturation of mammalian spermatozoa contrasts with the situation in invertebrates and lower vertebrates (such as teleosts and anuran amphibians), in which the spermatozoa leaving the testis already have full fertilising capacity (Yanagimachi, 1994).

1.3.i. Remodelling of the plasma membrane

As mentioned previously, many sperm maturational changes take place while the spermatozoa transit the epididymis. One major structural change that occurs to the sperm cells in the epididymis is the extensive remodelling of the plasma membrane. During passage of spermatozoa through the epididymis, remodelling of the plasma membrane is commensurate with acquisition of motility and fertilising capacity (Jones, 1998). During spermiogenesis, the insertion of newly synthesised membrane components coupled with polarised migration is the basis for the establishment of separate membrane domains that may be subdivided into the acrosome, postacrosome, midpiece and principal piece (Cowan and Myles, 1993).

A cell membrane basically consists of a bilayer arrangement of lipids that form spontaneously in an aqueous environment (Singer and Nicolson, 1972). Lipids constitute 50-60% of the mass of animal cell membranes with the remainder being mainly proteins. The most abundant group of lipids is the phospholipids and in addition to heterogeneity in the nature of the polar head groups, phospholipids also differ according to the length and degree of unsaturation of attached acyl fatty acids. The longer the acyl chain and the more *cis*-double bonds in it, the more distorted their shape becomes, an arrangement that favours a bilayer structure as opposed to a rigid micelle (Jones, 1998). Long-chain fatty acids are also more flexible and have higher fluidity than saturated ones, an important property for spermatozoa to possess for the membrane fusion events associated with fertilisation.

Another important feature of membrane phospholipids, is the way in which they are asymmetrically arranged in the bilayer, both laterally and transversely. This is due to the preference of different phospholipids to accumulate either on the cytoplasmic/inner side, or exoplasmic/outer side of the plasma membrane, which is of great functional relevance. Many transmembrane signalling systems are dependent on the presence of specific phospholipids. Protein kinase C within the cell requires phosphatidylserine for activation. Consequently it is located on the cytoplasmic side of the membrane (Devaux, 1992). Phosphatidylinositol is also predominately located on the cytoplasmic surface of the plasma membrane and it is the substrate for the formation of polyphosphinositides, which are crucial second messengers for triggering

intracellular Ca^{2+} release (Jones, 1998). The release of Ca^{2+} from intracellular stores, in addition to calcium influx from extracellular sources is important for the acrosome reaction in spermatozoa. In contrast, glycolipids which act as receptor molecules for cell-cell recognition and interaction with the extracellular matrix are generally found on the exoplasmic surface of the cell membrane (Jones, 1998).

As proteins are larger than lipids they tend to be interspersed among the lipids at approximately one protein molecule for 50 lipid molecules (Jones, 1998). Transmembrane proteins span the entire lipid bilayer of the membrane and as they form an integral part of the membrane they require detergents for solubilisation. Peripheral proteins bind weakly to both outside and inside layers of the membrane bilayer and can be dissociated easily by extremes of pH, reducing agents or high ionic strength medium. Consequently the presence of such membrane proteins is dependent on the nature of the environment to which the cell is exposed. A separate class of proteins consists of proteins restricted to the cytoplasmic face of the bilayer. The diverse nature of membrane-bound proteins and their difference in orientation to the lipid bilayer not only emphasises the asymmetry of the membrane, but also indicates how separate membrane domains can be easily differentiated from each other, as demonstrated in the formation of spermatozoa.

Sperm lipids possess the following unusual features: the presence of large amounts of plasmalogen phospholipids, a high content of polyunsaturated fatty acids which lends the membrane greater fluidity as mentioned previously and relatively low cholesterol. During the transit of spermatozoa through the epididymis, there is a decrease in the cellular content of phospholipids of up to 48% (Poulos *et al.*, 1975; Poulos *et al.*, 1973; Rana *et al.*, 1991; Scott *et al.*, 1967). However, due to the difficulties in obtaining pure sperm plasma membrane, the results obtained may not truly reflect the changes in phospholipid of the sperm plasma membrane, but rather the entire composition of the cell. Collectively though, the data indicates that the lipids in the plasma membranes of caudal spermatozoa differ in composition to those obtained from the testis. Detailed studies involving the use of whole sperm (Dawson and Scott, 1964; Devaux, 1992; Evans and Setchell, 1979; Grogan *et al.*, 1966) and purified plasma membrane (Nikolopoulou *et al.*, 1985; Parks and Hammerstedt, 1985) have established that phospholipid, cholesterol and bulk phospholipid-bound fatty acids, decline progressively during transit through different levels of the epididymis.

What is the purpose of selective loss of phospholipids?

Several ideas have been postulated, including the hypothesis that fatty acids derived from phospholipids provide an energy source for maturing spermatozoa, since exogenous energy sources are relatively low in epididymal fluid (Mann, 1964). However, this concept has not been proven. An additional proposal was the presence of active lipid exchange proteins that mediate the transfer of phospholipids from spermatozoa to membrane bound vesicles in epididymal fluid (Jones, 1998). However, if such exchange proteins existed it is likely that they would only affect those phospholipids present on the outer surface of the membrane. Another possibility, is related to the effect on the actual membrane properties in relation to its behaviour.

During epididymal maturation there is a decrease in the proportion of the phospholipid, phosphatidylethanolamine and saturated fatty acids, accompanied by an increase in unsaturated fatty acids, which theoretically should increase fluidity of the membrane. It has been suggested that membrane fluidity is a prerequisite for membrane fusion events (Israelachvili *et al.*, 1980). Data supporting this concept has demonstrated that certain lipids and free unsaturated fatty acids increase membrane fluidity and induce the acrosome reaction *in vitro* (Fleming *et al.*, 1982; Fleming and Yanagimachi, 1981; Meizel and Turner, 1983). Hall and colleagues (Hall *et al.*, 1991) demonstrated in rat sperm that the cholesterol/phospholipid and protein/cholesterol mass ratio, were highest in caput sperm plasma membrane and lowest in cauda sperm plasma membrane, whereas the total lipid/protein mass ratio remained relatively constant (Hall *et al.*, 1991). Using the measurement of calcium-dependent ATPase activity as a function of temperature, to measure changes in the sperm membrane physical state, they were able to indirectly correlate epididymal changes in membrane fluidity with the above mentioned changes in biochemical composition (Hall *et al.*, 1991).

As epididymal maturation leads to the increased ability to respond to physiological and pharmacological inducers of exocytosis, it is crucial that the sperm plasma membrane maintains some stabilisers to prevent the premature acrosome reaction (Nolan and Hammerstedt, 1997). Changes in cholesterol composition and the maintenance of an asymmetric distribution of transmembrane phospholipids, is believed to be very important for stabilisation of the membrane to withstand the storage conditions of mature spermatozoa in the cauda epididymis and to prevent premature acrosomal exocytosis (Nolan and Hammerstedt, 1997). The environment of the female tract provides conditions that promote efflux of cholesterol from the sperm plasma membrane in addition to membrane asymmetry (Nolan and Hammerstedt, 1997). The cholesterol-limited, lipid-asymmetric plasma membrane possesses a destabilised inner

leaflet that facilitates membrane fusion upon exposure to the appropriate egg coat receptors (Nolan and Hammerstedt, 1997). This led to the proposal of a mechanism by which the sperm membrane contains destabilisation components (e.g. highly unsaturated phosphatidylcholine) of its own to confer fusogenic potential, in addition to its stabilising components, such as cholesterol, whose responsibility is to maximise membrane integrity (Nolan and Hammerstedt, 1997). It would seem reasonable to suggest that the environment to which spermatozoa are exposed exercises some element of control over which of these factors are activated at a specific time.

In addition to changes in the lipid composition of the plasma membrane of spermatozoa, there are also many changes in protein composition of the membrane that are thought to have physiological significance. Available data indicates that the protein composition of rat sperm membranes alters considerably during the transport from testis to cauda epididymis and similarly the protein composition of rete testis fluid and cauda epididymal fluid were also found to be different (Jones *et al.*, 1983b). Testicular sperm appeared to possess mostly high molecular weight proteins, whereas those of the cauda were predominantly labelled with lower molecular weight proteins (Jones *et al.*, 1983b). The sperm plasma membrane has both membrane-bound and surface-adsorbed proteins when spermatozoa leave the testis (Yanagimachi, 1994) and references therein). Many changes occur in that these proteins may change their location, are masked or replaced progressively with other proteins. Monoclonal antibodies have been used to localise proteins and their changing locations during epididymal maturation (Phillips *et al.*, 1991). Many investigations have also been carried out using monoclonal antibodies, to identify which protein antigens have a potential role in fertilisation in a variety of species such as the rat (Shalgi *et al.*, 1990), mouse (Saling, 1986; Saling *et al.*, 1985a; Saling and Lakowski, 1985b; Saling *et al.*, 1983; Schmell *et al.*, 1982), hamster (Moore and Hartman, 1984) and guinea-pig (Primakoff *et al.*, 1985). However, the precise function of most sperm plasma membrane proteins is unknown. The immunolocalisation of several proteins to the rat sperm head of cells attached to the zona, suggested that different antigens in this region of the cell, participate in different aspects of fertilisation and also that during capacitation, there is either exposure of these antigens or they migrate to their site of action from the flagellum (Shalgi *et al.*, 1990).

There are also major changes in sperm plasma membrane glycoproteins during epididymal maturation. Using various types of lectins, data obtained have shown that some maturation-dependent sperm surface glycoproteins, co-migrated through the epididymis with glycoproteins present in epididymal fluid (Srivastava and Olson,

1991). Another study demonstrated that high concentrations of glycosyltransferases and glycosidases are present in epididymal luminal fluid and that these enzymes may be involved in the modification of rat sperm plasma membrane glycoproteins, during epididymal maturation (Tulsiani *et al.*, 1998). When spermatozoa attain full maturity, some surface glycoproteins are located over the entire sperm head , whereas other are restricted to the acrosomal or postacrosomal region of the head (Yanagimachi, 1994) and references therein). Some of these glycoproteins and other proteins are thought to function as stabilisers of the plasma membrane to prevent the premature exocytosis of the acrosome (Reynolds *et al.*, 1989; Thomas *et al.*, 1984). Adsorption and integration of several specific glycoproteins on or in the plasma membrane of the tail are also well documented (Yanagimachi, 1994) and references therein. It has been suggested that some surface glycoproteins located to the sperm tail could be involved in the prevention of premature hyperactivation. In addition, an epididymal protein referred to as DE, has been localised to the dorsal portion of the rat sperm head (Cameo *et al.*, 1986) and antibodies raised against this glycoprotein were shown to block the fertilising ability of rat spermatozoa (Cuasnicu *et al.*, 1984).

It is clear so far, that the epididymal secretions influence surface modifications during epididymal maturation (Hall and Killian, 1989; Srivastava and Olson, 1991). Incubation of either rat caput or corpus spermatozoa in caudal epididymal fluid was characterised by the loss of several polypeptides and the addition of a 24 kDa glycopolypeptide (Srivastava and Olson, 1991). Caput spermatozoa were also seen to gain an additional two glycopolypeptides of 32 and 33 kDa, following incubation in caudal luminal fluid (Srivastava and Olson, 1991). Although the changing pattern of protein secretion along the epididymis has been correlated with changes in surface protein on spermatozoa, most of the major proteins identified appear to play a homeostatic role in maintaining the epididymal milieu for spermatozoa (Dacheux *et al.*, 1998). However, a unique carbohydrate epitope attached to the peptide CD52, is synthesised in the epididymis and is subsequently inserted into the sperm membrane via a glycosylphosphatidylinositol anchor (Diekman *et al.*, 1999). This unique CD52 glycoform is localised to the entire sperm surface and has been shown to be related to human antibody-mediated infertility (Diekman *et al.*, 1999).

1.3.ii. Epididymal maturation of sperm structures other than the plasma membrane

During epididymal maturation, several changes occur to other structures of the spermatozoon. The distribution of antigens located to the outer acrosomal membrane is altered (Phillips *et al.*, 1991). Phillips *et al* (Phillips *et al.*, 1991), found that in addition to remodelling of the surface membrane of rat spermatozoa during epididymal maturation, there was also extensive processing of intracellular membrane antigens, highly active within the acrosome. Using monoclonal antibodies against the epitope 2D6, it was observed that spermatozoa from the distal caput, following freeze-thawing, were positive in the entire acrosomal region, indicating that the antibody was binding to an intraacrosomal antigen (Phillips *et al.*, 1991). Positivity for this antigen was not oberved in testicular or proximal caput spermatozoa (Phillips *et al.*, 1991). In contrast 2D6 McAb (monoclonal antibody) bound only to the postacrosomal and tail domains of corpus and cauda epididymal spermatozoa and freeze-thawing failed to expose intracellular antigens for 2D6 McAb (Phillips *et al.*, 1991). It was postulated that the 2D6 McAb actually recognised two different antigens, one located in the head of immature distal caput spermatozoa and a separate antigen acquired during maturation in the tail of caudal spermatozoa (Phillips *et al.*, 1991). The intraacrosomal antigen had not simply migrated from the acrosomal region to the tail, but a more favourable explanation was that it was actually masked during epididymal maturation and a 2D6 McAb bound antigen was gained in the tail region. Although the nature of these intracellular processing events has not yet been elucidated, it is possible that they have important consequences for membrane fusion and cell recognition during fertilisation (Phillips *et al.*, 1991).

The acrosome has been demonstrated to undergo gross morphological changes during epididymal maturation in some species such as the guinea pig, bush baby, pig-tailed monkey and marsupials (Yanagimachi, 1994) and references therein). Changes in the enzymes have also been observed including biochemical alterations in the proacrosin-acrosin system, during epididymal maturation of rat spermatozoa (Nagdas *et al.*, 1992). These changes involve the maturation-dependent appearance of several low molecular weight forms of proacrosin-acrosin (Nagdas *et al.*, 1992). During epididymal transit, the 52 kDa precursor form, proacrosin is partially converted to the low molecular weight components of 41, 34 and 31 KDa (Nagdas *et al.*, 1992). These studies indicate that a sperm cell must undergo important intra-acrosomal events during epididymal transit in order to become a functionally mature sperm cell (Nagdas *et al.*, 1992).

The subacrosomal cytoskeleton of sperm heads occupies the narrow space between the inner acrosomal membrane and the outer membrane of the nuclear envelope (Olson *et al.*, 1976) and is a prominent feature of rodent sperm with falciform-shaped heads, such as the rat, mouse and hamster (Eddy and O'Brien, 1994). In these species, the subacrosomal cytoskeleton is referred to as the perforatorium and in rat sperm it is a curved triangular rod anterior to the apex of the head (Eddy and O'Brien, 1994). However, the subacrosomal cytoskeleton is a minor component of the spatula-shaped heads present in sperm of most mammalian species, such as the human (Calvin and Bedford, 1971; Lalli and Clermont, 1981). The perforatorium becomes more resistant to solubilisation during epididymal transit, possibly due to extensive disulphide bond formation (Austin and Bishop, 1958; Calvin and Bedford, 1971; Olson *et al.*, 1976).

During epididymal maturation, it is important that sperm structures such as the chromatin, midpiece and tail become stabilised (Calvin and Bedford, 1971). Data has previously been generated indicating that this stabilisation is achieved mainly through the oxidation of thiol groups to disulfides (Bedford and Calvin, 1974a; Bedford and Calvin, 1974b; Pellicciari *et al.*, 1983). The suggestion of an association between epididymal sperm maturation and oxidation of sperm protein thiols, was based on the observations of increased resistance of chromatin to decondensation and reduced solubilisation of sperm tails by the detergent, sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) (which reduces disulfides to thiols), in spermatozoa undergoing maturation (Bedford and Calvin, 1974a; Bedford and Calvin, 1974b; Calvin and Bedford, 1971).

In a study on the thiol status of rat spermatozoa, using the fluorescent labeling agent monobromobimane, researchers were able to demonstrate a clear shift in the thiol status of rat spermatozoa as they underwent epididymal maturation (Shalgi *et al.*, 1989). Although the total amount of thiols and disulfides remained approximately the same throughout maturation (extending from the testicular region to the vas deferens), significant changes in the ratio of thiols to disulfides was observed (Shalgi *et al.*, 1989). In testicular sperm essentially all cysteine is in the reduced form, whereas in corpus sperm, thiols comprised about 75% of the total and this was reduced to only 25% in caudal and vas deferens spermatozoa (Shalgi *et al.*, 1989). Thus it was evident that the major oxidation of thiols to disulfides was found to occur in the epididymis between the corpus and cauda, supporting the idea that the number of disulphide bonds increase during epididymal maturation to stabilise various sperm structures. Further evidence comes from a study in which thiol oxidase activity was found to increase in activity in the epididymis, when compared with enzyme activity in the testis (Zirkin *et*

al., 1985). It was proposed that this enzyme may play a role in the formation and maintenance of sperm disulfide bonds (Zirkin *et al.*, 1985).

Cross-linking by disulphide bonds of nuclear protamines occurs as spermatozoa transit the epididymis and the resultant rigidity of the nuclei (heads) seems to facilitate sperm passage through the zona pellucida, by permitting the tail's thrust to be translated along the axis of the sperm head to the zona directly (Bedford, 1991). Similarly, disulfide linking of proteins of the outer dense fibres and fibrous sheath during sperm maturation (Bedford and Calvin, 1974a) may serve to increase the bending force generated by the axonemmae (Bedford and Hoskins, 1990).

1.3.iii. Acquisition of sperm motility

One phenomenon associated with epididymal maturation of spermatozoa is the acquisition of motility. Testicular sperm are immotile both within the testicular environment and when suspended in physiological salt solutions (Yanagimachi, 1994). Alternatively, mature caudal spermatozoa are fully motile when released into physiological salt solutions (Yanagimachi, 1994). Observations in our own laboratory have shown that when rat spermatozoa are released into BWW media, those from the caput are weakly motile, exhibiting sluggish movement or 'twitching', whereas those from the cauda are highly motile and exhibit energetic movement.

The incapacity of testicular sperm to exhibit motile behaviour appears to be due in part to immaturity of the plasma membrane, because they can move almost as actively as mature caudal sperm if they are demembranated and exposed to ATP, cAMP and magnesium ions (Yanagimachi, 1994) and references therein. However, as the testicular sperm did not respond as quickly to ATP/cAMP as the mature cauda epididymal sperm, it was postulated that epididymal maturation may involve modification of dynein ATPase (White and Volglmayr, 1986). This makes sense, as motility is not needed prior to ejaculation from the cauda epididymis and vas deferens. Consequently the epididymis is the ideal location for modification of the cellular systems necessary for the acquisition of motility.

The transfer of substances from the epididymal fluid also appears to influence the sperm's ability to exhibit motile behaviour, such as glycerol-3-phosphorylcholine and a forward motility protein (Yanagimachi, 1994) and references therein). Modifications in a cAMP-modulated protein kinase system, in addition to the development of the mechanism that keeps intracellular calcium low, appear to contribute to the sperm's acquisition of motility (Yanagimachi, 1994) and references therein).

1.3.iv. Site of acquisition of fertilising ability

Anatomically, the actual site where mammalian spermatozoa begin to acquire their ability to fertilise is species dependent. Whereas most acquire their fertilising potential by the time they reach the proximal cauda epididymis, in the boar it occurs in the caput and the rat it is in the distal potion of the corpus epididymis (Dacheux and Paquignon, 1980). However, such descriptions are not definitive, as the requirement for epididymal maturation to confer fertilising ability upon human spermatozoa appears to be flexible, as demonstrated by the production of fertile spermatozoa from humans who have had their vas deferens linked by anastomoses to the caput epididymis, thus completely bypassing the epididymis with the exception of the initial segment (Schoysman and Bedford, 1986; Silber, 1989). This is also supported by data indicating that when the epididymal tubule is blocked or absent, a small proportion of spermatozoa still acquire fertilising ability in more proximal regions than the cauda, or corpus of the epididymis (Bedford, 1994; Moore and Akhondi, 1996). Therefore, it appears that at least in the human, most of the epididymis does not have an essential role in the maturation process of spermatozoa. Such a flexible functional relationship between the epididymis and spermatozoa, does not appear to exist to any degree in most other mammalian species. Interestingly, Temple-Smith *et al* (Temple-Smith *et al.*, 1998) with the use of various surgical procedures demonstrated that rat spermatozoa were able to develop a level of maturation in the distal corpus and caudal epididymal regions sufficient to ascend the female tract and fertilise ova, thus indicating that exposure of rat spermatozoa to the distal caput and proximal corpus regions was not obligatory (Temple-Smith *et al.*, 1998).

1.3.v. Development of *in vitro* models for the study of epididymal sperm maturation

The anatomical nature of the epididymis leads to limitations in the experimental approaches available for the study of epididymal maturation. Micropuncture and cannulation studies (as reviewed in Setchell *et al.*, 1994) have been used to provide information related to the luminal milieu. However, such methods do not facilitate the study of the maturation of spermatozoa and their interactions with the epididymis.

Developments in the last two decades have led to an *in vitro* technique of incubating spermatozoa with *in vitro* cultured epididymal epithelial cells, as a means of assessing various aspects of epididymal maturation. Although such a technique does not completely mimic the *in vivo* environment of the epididymis, it allows the detailed study of interactions between the epididymal epithelium and spermatozoa, in addition to

various parameters of epididymal maturation, such as acquisition of motility and fertilising ability.

The potential for sperm maturation *in vitro* first arose when it was observed that by incubating crude cytoplasmic extracts of sperm-free rabbit epididymis from the distal corpus region, with rabbit corpus spermatozoa that had previously been incubated *in vitro* for 24 hours, it was possible to significantly increase the fertilising capacity of the epididymal spermatozoa (Orgebin-Crist and Jahad, 1979). Since then the *in vitro* culture of epididymal epithelium has developed considerably and has replaced the use of crude epididymal tissue for co-culture purposes with spermatozoa. In one such study, hamster spermatozoa recovered from the proximal and distal regions of the caput epididymis were incubated with 3 day old epithelial cells obtained from the proximal corpus region (Moore *et al.*, 1986). Although the spermatozoa were either immotile or exhibiting slight twitching, prior to co-culture with the epithelium, slow but persistent flagellar movement was observed, following incubation with the epithelial cells after 8 and 24 hours. Such motile characteristics were not observed in the control preparations, which included cells incubated with and without androgen supplementation (Moore *et al.*, 1986). It was thus postulated, that changes to the character of sperm motility during migration through the epididymis may be induced to a limited extent under *in vitro* conditions, when the secretory and absorptive functions of epithelial cells are sufficiently maintained (Moore *et al.*, 1986).

More recently, a significant improvement in motility in relation to straight-line velocity was observed in hamster cauda epididymal spermatozoa following co-culture *in vitro* with epididymal epithelial cell cultures (Samayawardhena and Moore, 1998). The magnitude of improvement in sperm motility was found to be correlated with the age of the epididymal epithelial cultures, the maximum motility improvement being exhibited when spermatozoa were co-incubated with cultures of 5 and 7 days old (Samayawardhena and Moore, 1998). It was suggested that the beneficial effects of epithelium were maximal during this time period as it is the time when androgen receptors of epithelial cells are maximally expressed in culture and thus epididymal secretory function is at its greatest (Moore *et al.*, 1998).

In addition to motility changes (Moore *et al.*, 1986; Samayawardhena and Moore, 1998), data has been obtained using a monoclonal antibody, indicating that surface changes that occur during epididymal maturation *in vivo* may also be elicited *in vitro* (Smith *et al.*, 1986). Hamster caput spermatozoa were found to be positive for a 34 kDa antigen localised to the postacrosomal region and annulus following co-incubation with epididymal epithelium obtained from the proximal corpus after incubation for 8 and 24 hours (Smith *et al.*, 1986). Interestingly, this antigen was found in epithelial

cells of the proximal corpus epididymis but not in the caput or inital segment regions (Smith *et al.*, 1986). In addition, the fact that this antigen was located to sperm in the specific regions of the postacrosome and annulus indicates a specific binding to the sperm surface (Smith *et al.*, 1986).

Culture of hamster spermatozoa with epididymal epithelium has also been demonstrated to increase their actual fertilising ability (Moore and Hartman, 1986). Epididymides of adult male hamsters were ligated at the junction between the distal corpus and proximal cauda region (Moore and Hartman, 1986). Three days after the ligation, spermatozoa were extracted from the distal corpus region and incubated in co-culture with epithelial cells from the proximal cauda epididymis (Moore and Hartman, 1986). Following six hours incubation under these conditions, the fertilising capacity of the spermatozoa was significantly increased, motility was maintained and the increase in sperm fertilising ability was also associated with elevated numbers of sperm binding to the zona pellucida *in vitro* (Moore and Hartman, 1986). Sperm fertilising ability remained low in spermatozoa that had been incubated in co-culture with either epithelial cultures without androgen supplementation, or 8-12 day old epithelial cells with fibroblast overgrowth, or without epithelial cells (Moore and Hartman, 1986). In addition, spermatozoa obtained from the ligated corpus epididymis, expressed greater progressive motility than the intact controls, but their fertilising ability remained very low (Moore and Hartman, 1986). The fact that the hamster spermatozoa were able to maintain motility and also undergo capacitation under these incubation conditions indicates that elements present in the culture of epididymal epithelium were sufficient to support the final stages of hamster sperm maturation (Moore and Hartman, 1986). These results support the notion, that under optimal conditions, sperm maturational events associated with the acquisition of fertilising ability, can be induced to some extent, by co-incubation with cultured epididymal epithelium *in vitro*.

Epididymal cultures from human tissue have also been used in the study of *in vitro* maturation of epididymal spermatozoa (Akhondi *et al.*, 1997; Moore *et al.*, 1992). Specific proteins were found to be synthesised by cauda epididymal epithelial cells cultured *in vitro* and when caput spermatozoa were co-incubated with the cultured epithelium, a significant increase in progressive motility and sperm binding to human zona pellucida was induced (Moore *et al.*, 1992). In a second study, it was observed that the extended survival of human spermatozoa, was associated with close apposition of spermatozoa, by the equatorial region, to the apical membrane of cultured caput/corpus epididymal epithelial cells (Akhondi *et al.*, 1997).

What are the potential applications for the use of *in vitro* culture of epididymal epithelium?

There are many possible applications for the use of *in vitro* co-culture systems employing epididymal epithelium with epididymal spermatozoa. Apart from being a valuable tool for studying the various processes associated with epididymal maturation and interaction between spermatozoa and the epididymis, there are also implications for its clinical application. The co-culture of human epididymal epithelial cells with epididymal spermatozoa, could provide an *in vitro* treatment for men with dysfunctional epididymides.

A particularly interesting concept is the use of such a system in the evaluation of the effects of reproductive toxicants on epididymal epithelial function. *In vitro* co-culture of epididymal epithelium with hamster epididymal spermatozoa has already been used with some success, as a means of determining the effects of a reproductive toxicant on epididymal epithelial function by investigating the motility and survival of epididymal spermatozoa following co-incubation (Samayawardhena and Moore, 1999). Hamster epididymal epithelial cultures were exposed to low concentrations of the reproductive toxicant, methoxy acetic acid, for 12 hours and then the culture medium was changed and washes carried out to remove traces of the toxicant (Samayawardhena and Moore, 1999). After 36 hours, epididymal spermatozoa were incubated with the cultures and sperm motility and viability was checked at variable intervals of incubation (Samayawardhena and Moore, 1999). Interestingly, even though there was no morphological change in the epithelium following treatment, there was a significant increase in the sperm mean straight line and curvilinear velocities, in addition to a dose-dependent decrease in sperm viability, when compared with spermatozoa co-cultured with the control untreated epithelium (Samayawardhena and Moore, 1999). These results indicate the potential use of this co-culture system as an investigative tool for reproductive toxicology.

1.4. Capacitation

The section of the female tract, where spermatozoa are deposited at ejaculation from the cauda epididymis and vas deferens, is species dependent. In many eutherian mammals, such as the rabbit, cow and primates, semen is deposited into the vagina (Yanagimachi, 1994). In other mammals, including the pig, horse and many rodents, the majority of the ejaculate either enters the uterus directly or is forced through the cervical canal during coitus (as reviewed in Yanagimachi, 1994). The rat belongs to the second group, in that the ejaculate is deposited directly into the cornua of the uterus and sperm gain access to the oviduct, within a few minutes post-ejaculation, as determined by oviductal flushings (Shalgi, 1991).

Freshly ejaculated spermatozoa do not have the immediate capacity to fertilise an oocyte as they must first reside in the female tract for a period of time (Chang, 1984). The physiological changes that occur in order to confer upon the sperm, full competence for fertilisation, are collectively referred to as capacitation (Chang, 1984). The credit for the discovery of capacitation must be claimed by Chang (Chang, 1951; Chang, 1955) and Austin (Austin, 1951; Austin, 1952), who published their experimental findings in the early 1950s. However, observations indicating the requirement for spermatozoa to reside in the female genital tract for a period of time, in order to acquire full competency for fertilisation, were first reported by Noyes, Finkle and Rocke in 1949 (Noyes, 1953). Noyes and colleagues had observed that rabbit spermatozoa collected from either semen or the vas deferens were incapable of fertilising oocytes in the oviduct, whereas those spermatozoa that had resided in the oviducts of donors for between 4 and 8 hours could do so (Noyes, 1953).

The definition for capacitation is shrouded by controversy and has been modified over the years. In general, it refers to the time period required *in vivo* (in the female tract), or *in vitro* (in incubation media), for spermatozoa to fully demonstrate the ability to fertilise an oocyte and encompasses all the biochemical and membranous changes that bring about this transformation in fertilising capacity. Subsequently, the ability of spermatozoa to undergo the acrosome reaction in response to contact with the zona pellucida, has been included in the definition (Florman and Babcock, 1991; Kopf and Gerton, 1991; Ward and Storey, 1984). However, not all researchers agree with the inclusion of the acrosome reaction in the definition of capacitation (Chang, 1984). Capacitation is a reversible process. It has been demonstrated that the treatment of capacitated spermatozoa with fluids such as seminal plasma, reduces their ability to fertilise oocytes and a continued incubation, or a longer residence in the female genital tract is required, to overcome the inhibition and 'recapacitate' the

spermatozoa (Bedford and Chang, 1962). As the acrosome reaction is an exocytotic event, evidently it cannot be stopped or reversed once it has been induced (Yanagimachi, 1994). Furthermore there are specific ionic requirements for the promotion and support of capacitation and the acrosome reaction. Consequently, these two factors led to the segregation of the two processes. It could be argued that, whether or not the acrosome reaction is included within the definition of capacitation is not of paramount importance, as it is certain that spermatozoa must undergo capacitation before they are capable of undergoing the zona-induced acrosome reaction. Therefore it is feasible to refer to the zona-induced acrosome reaction as the endpoint of capacitation, followed by fertilisation.

Another aspect of sperm capacitation, is the accompanied acquisition of a distinctive pattern of motility, referred to as hyperactivation. The various changes in motility collectively referred to as sperm hyperactivation are thought to be important for penetration of the zona pellucida. In a number of mammalian species, such motility changes have been correlated with the acquisition of the capacitated state (Yanagimachi, 1994). Reports have been published demonstrating the dissociation of capacitation and hyperactivation (Neill and Olds-Clarke, 1987), but complete independence of these two events has not been conclusively confirmed (Suarez, 1996). For the purpose of this review, sperm hyperactivation will be discussed during this section on capacitation, solely because both processes appear to occur in parallel.

The main purpose of capacitation is to ensure that spermatozoa reach the oocytes at the optimal time, in the appropriate state to fertilise the oocytes. This is achieved by finely controlling the rate of changes necessary to prime spermatozoa and by the activation of mechanisms needed for the subsequent acrosome reaction (de Lamirande *et al.*, 1997b). During this section the various changes that have been proposed to occur during this process will be discussed. However, one must bear in mind that most analyses carried out are *in vitro* studies and although they provide undoubtedly useful information for the dissection of the cellular processes taking place during capacitation, the conditions almost certainly do not mimic the *in vivo* situation. Therefore, although it is possible to capacitate most species of spermatozoa *in vitro* in a chemically defined media (Yanagimachi, 1994), it should not automatically be concluded that the regulation of this process is identical to the regulation of capacitation *in vivo*. The fact that spermatozoa of various species may be capacitated in various fluids such as defined media, heat-treated serum, oviductal fluid, follicular fluid or vitreous humour (Yanagimachi, 1994) suggests that specific aspects of capacitation can be initiated and controlled intrinsically by the sperm itself. However, the intrinsic nature of capacitation does not exclude the physiological relevance of

modulators of capacitation present in the male and female reproductive tracts. This underlines the importance of combining information regarding the intracellular regulation of capacitation, with the identification of stimulatory and inhibitory modulators of the process in the male and female genital tracts, thus improving the chances of elucidating which aspects of the *in vitro* model are likely to reflect the *in vivo* situation.

1.4.i. *In vivo* capacitation

In vivo, spermatozoa become capacitated as they migrate through the female reproductive tract. As mentioned previously, little is known of the *in vivo* situation, as data pertaining to it are scarce in comparison with *in vitro* studies relating to the phenomenon.

Proteins found in bovine seminal plasma have been shown to modulate sperm capacitation by high density lipoprotein (Therien *et al.*, 1997). These proteins bind to the surface of sperm after ejaculation and are thought to interact with heparin, apolipoprotein A-I and apolipoprotein A-I associated with high density lipoprotein (Therien *et al.*, 1997). Both heparin and high density lipoprotein are found in the female reproductive tract and have been implicated in sperm capacitation and the acrosome reaction. High density lipoprotein (HDL) increased levels of acrosome reactions in epididymal sperm and this was increased further, when cells were also incubated with bovine sperm proteins (Therien *et al.*, 1997). Similarly, bovine sperm proteins also enhanced the number of apolipoprotein A-I induced acrosome reactions (Therien *et al.*, 1997). No induction of the acrosome reaction in epididymal spermatozoa was observed when they were incubated with low density lipoproteins either with or without bovine sperm proteins (Therien *et al.*, 1997). Consequently, it was suggested that bovine sperm proteins may play a role in modulating the process of capacitation induced by heparin, HDL and apolipoprotein A-I (Therien *et al.*, 1997).

In the human, cervical mucus has been shown to induce sperm capacitation (Zinamen *et al.*, 1989) and hyperactivation (Zhu *et al.*, 1992; Zhu *et al.*, 1994), but not the acrosome reaction (Bielfeld *et al.*, 1991). This suggests that cervical mucus may play a role in the maturation of spermatozoa, but also serves to preserve sperm function in that it is not a physiological inducer of the acrosome reaction. It has also been proposed that the human endometrium may also play a role in sperm maturation as it secretes a 54 kDa sialic acid-recognising protein that binds to the plasma membrane of the sperm head *in vitro*, before but not after capacitation (Bannerjee and Chowdhury, 1994). Data has been published indicating that sperm-epithelial interactions may be an

important event during the residence of spermatozoa within the Fallopian tube, as human spermatozoa bind strongly to *in vitro* cultured Fallopian epithelial cells (Pacey *et al.*, 1995b; Pacey *et al.*, 1995a). In addition, Fallopian tube epithelial cells have also been found to exert positive effects on sperm viability and motility, in relation to their percentage motile population and hyperactivation status (Kervancioglu *et al.*, 1994). Interestingly, human spermatozoa incubated with cervical fluid, followed by incubation with Fallopian tube fluid *in vitro*, exhibited higher levels of hyperactivated motility, but reduced rates of acrosome reaction when exposed to follicular fluid, in comparison with those that had been exposed to follicular fluid without preincubation with the tubal fluid (Zhu *et al.*, 1994). These results suggest that human Fallopian tubal fluid may not only stimulate capacitation and hyperactivated motility, but also inhibit the cells to a certain extent to prevent the premature acrosome reaction.

The mechanisms relating to how the fluids of the female reproductive tract influence capacitation in spermatozoa are unclear, as are the mechanisms and functions of sperm-epithelial cell interactions (de Lamirande *et al.*, 1997b). A sialylated oligosaccharide similar to that found on fetuin, has been detected on the epithelium of the Fallopian tubes in hamsters and it also appeared to be recognised by sperm surface components, in addition to mediating the binding of spermatozoa to the tissue (Demott *et al.*, 1995). Demott *et al* (Demott *et al.*, 1995) provided further interesting observations when they demonstrated that the loss of specific fetuin- and sialic acid-recognising lectins from hamster sperm membranes, could be correlated with the release of the epithelium-bound spermatozoa from the Fallopian tube epithelium. Both these parameters were also correlated with the acquisition of hyperactivated motility (Demott *et al.*, 1995). However, the function of such sperm-epithelium interactions and membrane modifications remains to be elucidated.

The secretion of proteins from Fallopian tube epithelial cells has been implicated in the promotion of capacitation. Specific bovine Fallopian tube proteins have been found to bind strongly to spermatozoa and promote long term maintenance of motility (Lapointe and Sirard, 1996). The observation that the binding of these proteins to spermatozoa was calcium dependent, led to the investigators postulating that the function of the proteins may be to stabilise the cell membranes (Lapointe and Sirard, 1996). They would function by binding strongly to spermatozoa in the isthmus of the Fallopian tube, where calcium concentration is higher and then promote capacitation by being slowly removed from spermatozoa in the ampulla, where calcium concentration is reduced in comparison (Lapointe and Sirard, 1996). Paradoxically, it has also been proven that Fallopian tubal fluid contains proteins that could destabilise sperm membranes by promoting cholesterol efflux (Davis, 1982; Ehrenwald *et al.*, 1990), thus

increasing membrane fluidity in preparation for the membrane fusion events of fertilisation. Consequently, the Fallopian tube appears to provide a carefully balanced environment of negative and positive modulators of sperm capacitation, to ensure correct timing of all the changes that must occur in spermatozoa, in order for successful fertilisation to take place.

1.4.ii. Methods of evaluating sperm capacitation

Traditionally, there have been two main methods used for the evaluation of sperm capacitation status, the first being changes in chlortetracycline fluorescence pattern and the second is acrosome reaction status.

Chlortetracycline is an antibiotic, the fluorescence of which changes when it chelates membrane-associated divalent cations such as calcium (Hallett *et al.*, 1972). Using this probe it is possible to measure the percentage of non-capacitated, capacitated and acrosome-reacted spermatozoa in the same preparation (de Lamirande *et al.*, 1997b). This method of assessment for sperm capacitation status and the acrosome reaction was first used in the mouse (Ward and Storey, 1984). It has since been used in the human (DasGupta *et al.*, 1993; Lee *et al.*, 1987; Perry *et al.*, 1995), monkey (Kholkute *et al.*, 1990), bull (Fraser *et al.*, 1995), dog (Guerin *et al.*, 1999) and the rat (Oberlander *et al.*, 1996).

The evaluation of acrosome reactions induced with ligands that allow discrimination between those that are capacitated and those that are not, has often been used as a means of assessing the capacitation status of spermatozoa. Ideally it is preferred to use physiological ligands, ZP3 in particular, since it is this glycoprotein that acts as the natural receptor for capacitated spermatozoa on the surface of the zona pellucida and initiates the acrosome reaction. However, ZP3 is not easily available, consequently limiting its use for routine experimental purposes. Human recombinant ZP3 has been used successfully to induce the acrosome reaction in capacitated spermatozoa (Aitken *et al.*, 1995). However, it is not commercially available at this present time. Another physiological inducer of the acrosome reaction *in vitro* is progesterone (Cheng *et al.*, 1998; Cross, 1996), although one must bear in mind that it has also been demonstrated to capacitate human spermatozoa (Emiliozzi *et al.*, 1996; Foresta *et al.*, 1992; Uhler *et al.*, 1992). This introduces the possibility that progesterone could increase the population of capacitated spermatozoa in addition to inducing the acrosome reaction, thus affecting true estimations of 'naturally' occurring capacitated spermatozoa *in vitro*.

The calcium ionophore A23187, among others, has frequently been used to induce the acrosome reaction in spermatozoa as it causes calcium influx across the sperm plasma membrane. However, although the ionophore-induced acrosome reaction is associated with increased phosphoinositide metabolism and activation of phospholipase C (Bennett *et al.*, 1987; Ribbes *et al.*, 1987), it appears to differ structurally from the true physiological acrosome reaction (Watson *et al.*, 1992). It is also a potentially toxic compound and consequently the concentration of A23187 and the incubation conditions must be chosen very carefully. For example, concentrations of no more than 2.5 μM A23187, in the presence of albumin, were demonstrated to be most suitable to promote the acrosome reaction and maximal sperm/oocyte penetration, without exerting a major deleterious effect on sperm viability in human spermatozoa (Aitken *et al.*, 1993). Another agent that has been used for inducing the acrosome reaction is, lysophosphatidylcholine (Parrish *et al.*, 1988; Therien *et al.*, 1997; Yanagimachi and Suzuki, 1985). This compound is a membrane disturbing agent that has been detected in high concentrations in the bovine Fallopian tubes near where fertilisation takes place (Grippo *et al.*, 1994). Observations have indicated that it stimulates the fertilising ability of spermatozoa and induces changes on the zona pellucida and on the oolemma, thus promoting sperm-egg fusion (Riffo and Parraga, 1997). However, like A23187, lysophosphatidylcholine also has the potential to exert toxic effects upon spermatozoa (Jarvi *et al.*, 1993).

1.4.iii. Signal transduction during capacitation

As discussed earlier, *in vivo* capacitation studies are minimal in quantity, due to the inadequacy of ethical and practical experimental techniques. As a consequence, *in vitro* studies prevail as the most efficient and useful way of studying sperm capacitation events. The intrinsic nature of sperm capacitation and the validity of *in vitro* investigations into this phenomenon, may be validated to a large extent, by the fact that many species of spermatozoa undergo spontaneous capacitation in defined media, without requiring the addition of biological fluids. Thus a wealth of information regarding the molecular basis of *in vitro* sperm capacitation has been generated in many species. It is hoped that this knowledge may be pooled together with a view to providing a unified hypothesis regarding the regulation of capacitation at the molecular level.

Throughout this section, the various media constituents and conditions required for the *in vitro* support of capacitation will be discussed in detail. A useful way to view *in vitro* capacitating media is to look upon it as an alternative to female genital tract

fluid. As a consequence interesting questions are raised, such as, in what way do certain conditions required for capacitating sperm *in vitro* reflect the conditions of the female tract (for example, pH or Ca^{2+} levels)? Similarly, albumin is required for supporting *in vitro* capacitation in most species, so what albumin-like substances exist in the female tract that could function in the same way? By thinking in this way, it keeps in mind that as useful as *in vitro* studies are for dissecting the intracellular signalling and processes of capacitation, it is crucial to appreciate how such studies may relate to the *in vivo* situation.

The media constituents, serum albumin, Ca^{2+} and HCO_3^- have been found to play critical regulating roles in all species studied so far (Kopf *et al.*, 1999). The first of these, serum albumin (mostly bovine serum albumin; BSA), is thought to support *in vitro* capacitation by acting as a sink for the removal of cholesterol from the sperm plasma membrane (Cross, 1998; Go and Wolf, 1985; Langlais and Roberts, 1985). Cholesterol removal from the sperm plasma membrane was first correlated with capacitation in 1980 (Davis *et al.*, 1980a) and such changes are thought to account for the increase in membrane fluidity associated with this maturational event (Wolf *et al.*, 1986).

How does cholesterol removal from the sperm plasma membrane, mediate capacitation?

The increase in phosphorylation of tyrosine residues, on specific sperm proteins, is highly correlated with the acquisition of the capacitated state (Aitken *et al.*, 1996a; Aitken *et al.*, 1995; Carrera *et al.*, 1996; Emiliozzi and Fenichel, 1997; Galantino-Homer *et al.*, 1997; Leclerc *et al.*, 1996; Luconi *et al.*, 1996; Visconti *et al.*, 1995a; Visconti *et al.*, 1995b) and the concomitant expression of hyperactivated motility (Mahoney and Gwathmey, 1999; Si, 1997). This process is regulated by cAMP at the level of PKA through a unique mode of signal transduction cross-talk (Visconti *et al.*, 1995b) (Refer to Fig. 1.4.)

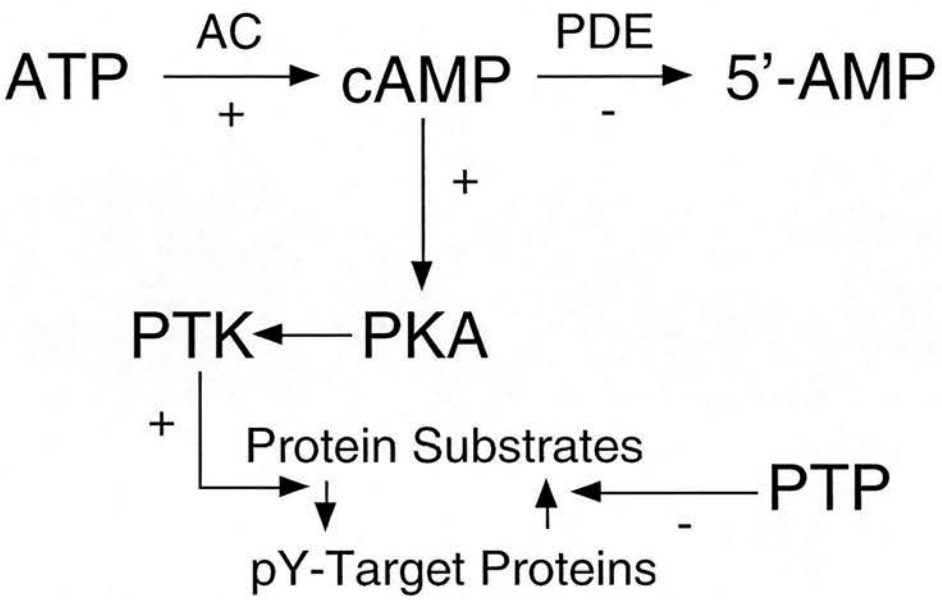


Figure 1.4. This diagram represents a simplified cartoon of the signal transduction pathways involved in the process of tyrosine phosphorylation. ATP is converted into cAMP by the action of adenylyl cyclase (AC). The breakdown of cAMP into 5'-AMP is brought about by the enzymes referred to as phosphodiesterases (PDE). cAMP activates PKA which in turn stimulates protein tyrosine kinases (PTK) to act upon protein substrates resulting in the phosphorylation of tyrosine residues (pY) on target proteins. The dephosphorylation of PKA phosphorylated proteins is induced by protein tyrosine phosphatases (PTP).

It is presumed that the presence of cholesterol in seminal plasma inhibits cholesterol efflux from the sperm plasma membrane, thus accounting for the inhibitory effects of seminal plasma on human sperm capacitation (Cross, 1998). In addition, to providing a sink for cholesterol efflux (Cross, 1998; Go and Wolf, 1985; Langlais and Roberts, 1985), BSA has also been proven to be vital for the capacitation-associated changes in tyrosine phosphorylation (Visconti *et al.*, 1995b).

A recent study carried out in the mouse by Kopf's lab, has confirmed further, the involvement of BSA in the support of the signal transduction cascades leading to capacitation (Visconti *et al.*, 1999a). In addition these data support the existence of a correlation between cholesterol efflux and the activation of membrane and transmembrane signalling events leading to the activation of tyrosine kinase second messenger systems (Visconti *et al.*, 1999a). Their observations demonstrated that the cholesterol-binding heptasaccharides, methyl-β-cyclodextrin and OH-propyl-β-

cyclodextrin were capable of completely compensating for the lack of BSA in the incubating media, in relation to the capacitation-associated changes in tyrosine phosphorylation and sperm capacitation itself, as measured by the ability of the zona pellucida to induce the acrosome reaction (Visconti *et al.*, 1999a). Their ability to increase tyrosine phosphorylation was also correlated with their cholesterol-binding efficiencies, as exemplified by the observation that preincubation of the compounds with cholesterol-SO_4, to saturate their binding sites, resulted in the loss of their ability to stimulate tyrosine phosphorylation (Visconti *et al.*, 1999a). The effect of these compounds on sperm protein tyrosine phosphorylation was both $NaHCO_3$ and PKA-dependent (Visconti *et al.*, 1999a). Further confirmation for such a model came from the publication of a similar study in human sperm, whereby almost identical observations to those in the mouse were obtained (Osheroff *et al.*, 1999).

Interestingly, HDL, another cholesterol-binding protein which is found in the female reproductive tract, was also found to support the capacitation-associated increase in tyrosine phosphorylation, through a cAMP-dependent pathway, in mouse spermatozoa (Visconti *et al.*, 1999b). In contrast, proteins that do not interact with cholesterol had no effect on tyrosine phosphorylation (Visconti *et al.*, 1999b). HDL was also found to support capacitation as assessed by *in vitro* fertilisation (IVF) (Visconti *et al.*, 1999b). Collectively, these findings provide a persuasive case for the existence of an association between cholesterol efflux, cholesterol-binding substances present in the female genital tract and the activation of tyrosine kinase second messenger systems leading to tyrosine phosphorylation and capacitation.

Extracellular Ca^{2+} is important for several sperm functions and capacitation is thought to be dependent upon the presence of this cation in mammalian spermatozoa (DasGupta *et al.*, 1993; Visconti *et al.*, 1995a). In addition Ca^{2+} is of central importance in the regulation of mammalian sperm motility (as reviewed in Lindemann and Kanous, 1989). However, there is a great deal of ambiguity associated with the precise role of Ca^{2+} in capacitation. This is mainly due to the well established role of calcium in the acrosome reaction and the difficulty involved in the division of capacitation from both this exocytotic event and hyperactivation.

It has been demonstrated in the mouse that calcium is crucial for the capacitation-associated changes in tyrosine phosphorylation and capacitation, as exemplified by the inhibitory effect of Ca^{2+}-free media on these two processes in the mouse (Visconti *et al.*, 1995b). In addition, capacitation has been associated with an increase in intracellular calcium in several species (Baldi *et al.*, 1991; Coronel and Lardy, 1987; White and Aitken, 1989; Zhou *et al.*, 1990). Published observations also indicate that

Ca^{2+} activates sperm adenylyl cyclase (Hyne and Garbers, 1979a; Kopf and Vacquier, 1984). However, the effect of Ca^{2+} on adenylyl cyclases can vary, depending on the type of adenylyl cyclase, of which there are several. Calcium is known to stimulate the activity of type I, III and VIII adenylyl cyclases, but it actually inhibits types V and VI (Cooper *et al.*, 1995). Therefore its effect on sperm adenylyl cyclases would depend upon which types predominated within the cell. It is thought that oscillations in cellular cAMP levels arise because of feedback inhibition of adenylyl cyclase by calcium (Cooper *et al.*, 1995). Additionally, activation of adenylyl cyclases by Ca^{2+} is generally seen at levels higher than the physiological concentration of Ca^{2+} (Lindemann and Kanous, 1989) and references therein). In studies of rat spermatozoa, no effect of Ca^{2+} on adenylyl cyclase was observed (Stengel and Hanoune, 1984).

Calcium is also known to stimulate the activity of a calmodulin dependent cyclic nucleotide phosphodiesterase, associated with demembranated rat caudal epididymal spermatozoa (Wasco and Orr, 1984), which could result in a reduction in intracellular cAMP levels. Consequently, the equivocal nature of the data creates an element of uncertainty as to the importance of Ca^{2+} as a regulator of adenylyl cyclase activity.

In contrast with observations in the mouse, Carrera *et al* (Carrera *et al.*, 1996) found that calcium actually induced the dephosphorylation of human sperm proteins and this was calmodulin-dependent which suggested that calcineurin was involved. In a separate study, the inhibitory effect of extracellular calcium on tyrosine phosphorylation in human spermatozoa was observed again (Luconi *et al.*, 1996). When tyrosine phosphorylation levels were suppressed by the tyrosine kinase inhibitor erbstatin, the progesterone-induced acrosome reaction was maintained (Luconi *et al.*, 1996). Yet, when spermatozoa were incubated in media devoid of calcium, tyrosine phosphorylation levels remained high but the progesterone-induced acrosome reaction was significantly compromised (Luconi *et al.*, 1996). Incubation with either the calcium ionophore A23187, or the Ca^{2+}-ATPase inhibitor thapsigargin, which promotes Ca^{2+} influx in human sperm, led to lower tyrosine phosphorylation levels (Luconi *et al.*, 1996).

The researchers concluded that tyrosine phosphorylation must be kept at a low level in the external environment of spermatozoa, in order to obtain a response to progesterone culminating in the acrosome reaction (Luconi *et al.*, 1996). However, conflicting reports have been generated by Aitken's lab whereby it was observed that A23187 actually enhanced tyrosine phosphorylation of human spermatozoa during capacitation (Aitken *et al.*, 1995). Further contrasting observations were provided by a study carried out on human spermatozoa whereby $CaCl_2$ removal from the incubation media, not only enhanced tyrosine phosphorylation, but also failed to impair the

induced acrosome reaction (Emiliozzi and Fenichel, 1997). Kopf's laboratory also demonstrated in the mouse, that inhibition of capacitation-associated tyrosine phosphorylation by Ca^{2+}-free conditions was completely reversed by the addition of phosphodiesterase inhibitors and cAMP analogues (Visconti *et al.*, 1995b). In addition, under these conditions, the ability of the spermatozoa to undergo capacitation was also redeemed, as assessed by chlortetracycline analysis and induction of the acrosome reaction by zona pellucida, even though the media was devoid of calcium (Visconti *et al.*, 1995b).

Several anomalies arise from these conflicting observations. Firstly, many studies fail to experimentally separate capacitation and the progesterone-induced acrosome reaction. Further confusion is provided by the multi-effects of progesterone in addition to its role as an inducer of the acrosome reaction. Progesterone has been shown to stimulate capacitation (Emiliozzi *et al.*, 1996; Foresta *et al.*, 1992; Uhler *et al.*, 1992) and tyrosine phosphorylation (Luconi *et al.*, 1995; Martinez *et al.*, 1999; Tesarik *et al.*, 1993b) in human spermatozoa. During the Luconi study in question, spermatozoa were incubated with progesterone for one hour (Luconi *et al.*, 1996), a sufficient amount of time to raise the possibility that progesterone may exert additional effects on the spermatozoa besides the induction of the acrosome reaction. An interesting concept to investigate would be to incubate spermatozoa in calcium free media and then add Ca^{2+} at the same time as progesterone, in an attempt to determine whether or not calcium was required for capacitation, or the acrosome reaction or indeed both.

Ca^{2+} is also known to play an important regulatory role in the maintenance of sperm motility. The initiation and maintenance of sperm motility is known to involve the cAMP-dependent phosphorylation of specific proteins (Lindemann and Kanous, 1989; San Agustin and Witman, 1994; Tash and Means, 1983). Moreover, a role for tyrosine kinases in the initiation of flagellar movement has been demonstrated in rainbow trout sperm (Hayashi *et al.*, 1987). Sperm motility was also found to be inhibited by calcium and this was directly correlated with the inhibition of protein phosphorylation via the calmodulin-dependent protein phosphatase calcineurin (Tash *et al.*, 1988). Thus it appears that calcium and cAMP are the major internal regulatory messengers interacting with the flagellum to activate (cAMP), deactivate (Ca^{2+}) and modify sperm swimming by direct action on the flagellar apparatus (Lindemann and Kanous, 1989). However, there are obviously external controls that regulate these internal messengers some of which are detailed in Table 1.3.

Agent	Source	Action
Spermine and spermidine	Seminal fluid	Activates adenylyl cyclase
Low molecular weight 'quiescence' factor	Epididymal fluid	Induces quiescence and inhibits the acrosome reaction.
Heparin	Mast cells and other sources.	Triggers capacitation and Ca^{2+} uptake
Acetylcholine and adenosine	Nerve and muscle	Bind to specific receptors present in sperm membrane
Low molecular weight motility-stimulating factor	Seminal plasma	Stimulates adenylyl cyclase activity and motility
cAMP	Female reproductive tract	Phosphorylates external membrane proteins
Thyroxine and triiodothyronine	Thyroid gland	Activates adenylyl cyclase

Table 1.3. Physiological agents which have been reported to regulate sperm motility. Adapted from (Lindemann and Kanous, 1989) and references therein.

Evidently calcium plays a significant role in the regulation of motility, cAMP-mediated tyrosine phosphorylation, capacitation and the acrosome reaction in spermatozoa. An intriguing possibility is that calcium availability varies in different regions of the female genital tract, thus providing a regulating mechanism during sperm transit to ensure the correct time of capacitation-associated events, so that spermatozoa only reach full maturation within the vicinity of the oocyte.

The requirement for HCO_3^- in capacitation has been established in several mammalian species including the mouse (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Visconti *et al.*, 1995a), hamster (Boatman and Robbins, 1991) and human (Aitken *et al.*, 1998b). In addition, the presence of HCO_3^- has been proven to be vital for the capacitation-associated changes in tyrosine phosphorylation (Aitken *et al.*, 1998b; Carrera *et al.*, 1996; Galantino-Homer *et al.*, 1997; Visconti *et al.*, 1995a).

The role of cAMP in the regulation of tyrosine phosphorylation is well documented in various species (Aitken *et al.*, 1998a; Bookbinder *et al.*, 1991; Brandt and Hoskins, 1980; Kalab *et al.*, 1998; Leclerc *et al.*, 1996; MacLeod *et al.*, 1994; Mahoney and Gwathmey, 1999; Tash and Means, 1983; Vijayaraghavan *et al.*, 1997a; Visconti *et al.*, 1995a; Visconti and Kopf, 1998; Visconti *et al.*, 1995b). Moreover, one of the best known intracellular activators of sperm adenylyl cyclase is believed to be bicarbonate (Okamura *et al.*, 1991; Okamura *et al.*, 1985). This is supported further by the inhibitory effect of HCO_3^--free media on tyrosine phosphorylation and capacitation in the mouse (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b). Interestingly, this inhibition of tyrosine phosphorylation and capacitation could be completely reversed under HCO_3^--free conditions, if the spermatozoa were treated with active cAMP analogues in combination with phosphodiesterase inhibitors (Visconti *et al.*, 1995b). Visconti *et al.*, 1999, also established in the hamster that $NaHCO_3^-$ was required for both tyrosine phosphorylation and capacitation. In agreement with their observations in the mouse, the inhibitory effect of HCO_3^--free conditions on these two processes was reversed by the presence of dbcAMP and the phosphodiesterase inhibitor IBMX (Visconti *et al.*, 1999). The group postulated that although tyrosine phosphorylation is necessary for capacitation, it is not sufficient for the completion of capacitation (Visconti *et al.*, 1999), a hypothesis supported further by data generated in human spermatozoa (Emiliozzi and Fenichel, 1997).

In hamster sperm, HCO_3^--free incubation conditions were found to inhibit the phorbol 12-myristate 13-acetate (PMA) stimulated elevation of [cAMP]i (Visconti *et al.*, 1990). In addition to stimulating [cAMP]i accumulation (Visconti *et al.*, 1990), phorbol esters such as PMA are also known to induce a $O_2^{\cdot-}$ burst in human spermatozoa (Aitken and Buckingham, 1992b; Aitken *et al.*, 1992a). In keeping with this observation, HCO_3^--free conditions were also found to inhibit ROS generation, tyrosine phosphorylation and the capacity to generate normal calcium transients in response to progesterone in human spermatozoa (Aitken *et al.*, 1998b).

Aitken's lab demonstrated that inhibition of tyrosine phosphorylation in human spermatozoa under HCO_3^--free conditions could be reversed, if the extracellular pH was raised to 8.4 (Aitken *et al.*, 1998b), thus implicating an association between HCO_3^-

and intracellular pH maintenance. The movement of HCO_3^- has been proposed to play a role in the known increase in intracellular pH that is observed during capacitation (Cross, 1998; Uguz *et al.*, 1994; Zeng *et al.*, 1996).

Changes in intracellular pH are involved in the regulation of capacitation and two acid efflux mechanisms have been identified in mouse sperm which could be involved in this process (Zeng *et al.*, 1996). Increases in intracellular pH associated with heparin-induced capacitation were not inhibited by the PKA antagonist Rp-cAMP (Uguz *et al.*, 1994). However, this antagonist can block capacitation thus indicating that a PKA regulatory pathway functions either parallel to, or downstream of pathways activated as a consequence of changes in intracellular pH. For example it is possible that changes in pH could modulate capacitation via the activation/inhibition of protein phosphatases downstream of PKA (refer to Fig. 1.4.). It has also been postulated that pH may have a role in the maintenance of the phosphorylated condition of the axoneme, possibly by influencing the reaction rates of the kinase(s), phosphatase(s) or phosphodiesterase(s) of mammalian spermatozoa (Lindemann and Kanous, 1989).

HCO_3^- is also required for the initiation and maintenance of hyperactivated motility in the spermatozoa of many mammalian species (Si, 1996; Si, 1997). This anion is thought to stimulate hyperactivation of spermatozoa, by elevating [cAMP]i through the direct stimulation of adenylyl cyclase (Si, 1996; Si, 1997). HCO_3^- has also been implicated in the progesterone-induced human sperm acrosome reaction (Sabeur and Meizel, 1995). Their observations indicated that HCO_3^- transport and HCO_3^- stimulation of adenylyl cyclase are involved in the mechanism of the progesterone-initiated human acrosome reaction (Sabeur and Meizel, 1995). Similarly, the availability of HCO_3^- in the extracellular media was requisite for human spermatozoa to generate normal calcium transients in response to progesterone (Aitken *et al.*, 1998b).

1.4.iv. Reactive oxygen species (ROS): their role in capacitation

An atom or group of atoms possessing an odd (unpaired) electron is called a free radical and they are extremely reactive due to their susceptibility to gaining another electron (Morrison and Boyd, 1992). More energy is required for the maintenance of two separate species, each with an unpaired electron, than for them to combine and share electrons, such that a full molecular orbital is established along with a covalent bond, hence their high levels of reactivity (Aitken and Fisher, 1994). Reactive oxygen species (ROS) fit in the category of free radicals and they originate from oxygen and enzymatic reactions (Halliwell and Gutteridge, 1989).

The controlled and well-timed generation of very low amounts of ROS appears to be required for spermatozoa to acquire their full fertilizing ability (as reviewed in Aitken and Fisher, 1994; de Lamirande and Gagnon, 1999; de Lamirande *et al.*, 1997a; de Lamirande *et al.*, 1997b). The ROS most often reported to affect sperm function are the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2, from the dismutation of $O_2^{\cdot-}$ by the action of superoxide dismutase; SOD) and the hydroxyl radical ($^{\cdot}OH$, from an iron -catalysed reaction involving H_2O_2) (Aitken *et al.*, 1993b; Aitken and Clarkson, 1987a; Alvarez *et al.*, 1987; de Lamirande and Gagnon, 1992a; de Lamirande and Gagnon, 1992b; Griveau *et al.*, 1995a).

The generation of ROS by mammalian spermatozoa was first described in 1943 by the andrologist John MacLeod who suggested that human spermatozoa were capable of generating H_2O_2 (Macleod, 1943). Since then ROS generation by spermatozoa has been observed in many species such as the bull (Tosic and Walton, 1946), mouse (Alvarez and Storey, 1984; Fisher and Aitken, 1997), rabbit (Alvarez and Storey, 1982), guinea pig, hamster, rat and human (Fisher and Aitken, 1997). In animal models including the rat, hamster, guinea pig and mouse, the generation of $O_2^{\cdot-}$ by precursor germ cells triggered by NADPH appears to be inversely correlated with the stage of cell differentiation, with the highest levels being measured in pachytene spermatocytes (Fisher and Aitken, 1997). Fisher and Aitken (Fisher and Aitken, 1997) also demonstrated that NADPH-induced $O_2^{\cdot-}$ generation was greater in immature caput epididymal spermatozoa than those obtained from the cauda in all species evaluated. It is therefore evident that ROS production by spermatozoa begins very early on in their maturational development. Fertile spermatozoa produce very low levels of ROS in semen or following a Percoll wash, but will initiate ROS generation at the time of capacitation (Aitken *et al.*, 1995; de Lamirande and Gagnon, 1993a; de Lamirande and Gagnon, 1993b; de Lamirande and Gagnon, 1995).

The existence of at least two enzymatic systems responsible for $O_2^{\cdot-}$ generation have been proposed: the first of which is a diaphorase (NADH-dependent oxido-reductase) (Gavella and Lipovac, 1992; Gavella *et al.*, 1995) and the second is an oxidase at the level of the plasma membrane (Aitken and Clarkson, 1987a; Aitken and Vernet, 1998c; de Lamirande and Gagnon, 1995a; Griveau and Le Lannou, 1997a). The existence of the second system is supported by the observation that NADPH induces an increase in $O_2^{\cdot-}$ production resulting in a decrease in sperm motility, hyperactivation and capacitation which are prevented by the combination of SOD and catalase (Griveau and Le Lannou, 1997a). It is believed that this membrane oxidase would normally be activated only at the time of capacitation and/or the acrosome reaction (Aitken *et al.*, 1996a; Aitken *et al.*, 1997; Aitken *et al.*, 1995; de Lamirande and

Gagnon, 1995; de Lamirande *et al.*, 1997a; de Lamirande *et al.*, 1997b; Leclerc *et al.*, 1997). Although the nature of such an oxidase remains elusive, it is thought to share similarities with the NADPH oxidase of neutrophils. This hypothesis has arisen from the fact it appears to be located at the level of the plasma membrane and its activity is reduced in the presence of inhibitors of the neutrophil NADPH oxidase, such as diphenyliodonium and lapachol (Aitken *et al.*, 1997; de Lamirande *et al.*, 1997b; Leclerc *et al.*, 1997). However, the proposed sperm NADPH oxidase differs from the oxidase of neutrophils in that activity is much lower (de Lamirande and Gagnon, 1995; de Lamirande *et al.*, 1998a). Moreover they seem to differ in that tyrosine phosphorylation of specific proteins is a prerequisite for the oxidative burst in neutrophils (Fialkow and Chan, 1993), but a consequence of ROS production in spermatozoa (Leclerc *et al.*, 1997).

Spermatozoa and seminal plasma possess ROS scavenging systems for the regulation of sperm ROS production. These include the following enzymes and enzyme-like/scavenging substances: SOD, catalase, the glutathione peroxidase/reductase system, phospholipid hydroperoxide glutathione peroxidase, vitamins E and C, ubiquinol-10, glutathione, taurine and hypotaurine (as reviewed in de Lamirande and Gagnon, 1999). Interestingly, the mRNAs for SOD and the glutathione peroxidase/reductase system are differentially expressed in the various regions of the rat epididymis, suggesting that the requirement for antioxidants may vary during epididymal maturation (Zini and Schlegel, 1997).

As mentioned previously, protein tyrosine phosphorylation and capacitation appear to be under regulation of a cAMP/PKA pathway. However, there is now evidence that ROS also play an important role in the regulation of the tyrosine phosphorylated events associated with capacitation (Aitken *et al.*, 1996a; Aitken *et al.*, 1995; Leclerc *et al.*, 1997). A suitable example demonstrating a link between tyrosine phosphorylation, capacitation and ROS was demonstrated by the behaviour of human spermatozoa incubated under HCO_3^--free conditions (Aitken *et al.*, 1998b). Under such conditions although the spermatozoa appear to be fully motile, their ability to generate ROS is compromised as is their expression of tyrosine phosphorylation (Aitken *et al.*, 1998b). As a consequence, they were unable to complete capacitation or exhibit functional responses to physiological agonists such as progesterone (Aitken *et al.*, 1998b). This effect was found to be associated with a fall in pH and if the cytoplasmic pH of the spermatozoa was buffered back to within the normal range, their ability to generate ROS, exhibit tyrosine phosphorylation and functionally respond to progesterone was restored (Aitken *et al.*, 1998b).

A direct involvement of ROS (H_2O_2 in particular), with tyrosine phosphorylation and capacitation was demonstrated when catalase was found to suppress both these processes (Aitken *et al.*, 1995). Incubating human spermatozoa with low doses of H_2O_2, or stimulation of intracellular H_2O_2 production by the addition of NADPH was found to accelerate capacitation (Aitken *et al.*, 1995). Similarly, exposure of hamster spermatozoa to low concentrations of H_2O_2 has been shown to enhance capacitation while catalase has been shown to disrupt it (Bize *et al.*, 1991).

In contrast with Aitken's observations, Claude Gagnon's group postulate that $O_2^{\cdot-}$ plays the most important role in regards to human sperm capacitation. They observed that exogenously added $O_2^{\cdot-}$ (xanthine and xanthine oxidase in the presence of catalase) promotes this process and SOD actually prevents sperm capacitation triggered by $O_2^{\cdot-}$, progesterone and biological fluids (de Lamirande *et al.*, 1993; de Lamirande and Gagnon, 1993a; de Lamirande and Gagnon, 1993b; de Lamirande *et al.*, 1998a). SOD is responsible for the dismutation of $O_2^{\cdot-}$ into H_2O_2. Therefore these observations indicate that H_2O_2 exerts an inhibitory action towards capacitation. These slightly conflicting reports probably arise from differences in experimental conditions. For example, the use of foetal cord serum in the incubation media employed by Gagnon's group might contain factors capable of stimulating $O_2^{\cdot-}$ production that are missing from the simple defined culture media used by most gamete biologists. Such factors aside, these observations are consistent in suggesting that ROS play an important role in capacitation and its associated changes in tyrosine phosphorylation. This hypothesis is confirmed further by additional reports suggesting a modulating role for ROS in capacitation in the hamster (Bize *et al.*, 1991), mouse (de Lamirande *et al.*, 1997a) and the bull (Blondin *et al.*, 1997; Goyette *et al.*, 1998; O'Flaherty *et al.*, 1997).

Spermatozoa themselves are the source of ROS production associated with capacitation (de Lamirande and Gagnon, 1995) and low levels are sufficient for this process (de Lamirande *et al.*, 1998a). It has been observed that $O_2^{\cdot-}$ production is initiated at the beginning of the capacitation period, peaks 15-25 minutes later and slowly decreases from then on (de Lamirande and Gagnon, 1995a; de Lamirande *et al.*, 1998a). Therefore an increase in $O_2^{\cdot-}$ production precedes the other processes associated with the phenomena of capacitation including sperm hyperactivation (between 1-3 hours), protein tyrosine phosphorylation (from 1 hour) and capacitation itself (progressive increase over 6 hours). An interesting published report revealed that $O_2^{\cdot-}$ generation is only required for the first 30 minutes of incubation under capacitating conditions, indicating that $O_2^{\cdot-}$ initiates an early chain of events, but is not

needed for the subsequent steps leading to the attainment of the capacitated state (de Lamirande *et al.*, 1998).

There are several theories relating to the mechanism of ROS action during sperm capacitation. There are indications that ROS may actually increase intracellular cAMP levels, since treatment of spermatozoa with low concentrations of xanthine and xanthine oxidase led to an increase in sperm intracellular cAMP concentrations and capacitation, both of which were prevented by SOD (Zhang and Zheng, 1996). Similarly, stimulation of sperm $O_2^{\cdot-}$ with NADPH was associated with a three-fold increase in intracellular cAMP concentration (Aitken *et al.*, 1998a). The mechanisms by which ROS stimulate capacitation-associated tyrosine phosphorylation could also be related to both inhibition of tyrosine phosphatase activity and stimulation of tyrosine kinase activity. Tyrosine phosphatases possess a conserved cysteine residue in their catalytic domain that must remain in a reduced state for expression of maximal activity (Aitken and Vernet, 1998). Exposure of this class of enzyme to H_2O_2 led to the suppression of tyrosine phosphatase activity (Hecht and Zick, 1992). H_2O_2 has also been implicated in the direct activation of tyrosine kinase activity in connection with such molecules as Lck, PLCγ or NF-κB (Kaul and Forman, 1996; Monteiro and Stern, 1996).

Sperm capacitation is associated with increased membrane fluidity (Yanagimachi, 1994) and although high levels of ROS cause lipid peroxidation which subsequently decreases membrane fluidity (Halliwell and Gutteridge, 1989), caffeine has been shown to increase $O_2^{\cdot-}$ production, decrease SOD activity and increase membrane fluidity of spermatozoa (Sinha *et al.*, 1993). Oxidation of protein sulfhydryl groups in spermatozoa by ROS also enables an easy and reversible means for switching signal transduction enzymes such as protein kinase C on (Gopalakrishna and Anderson, 1989) and tyrosine phosphatases off (Hecht and Zick, 1992).

An important aspect of sperm ROS generation is the fact that they are pernicious molecules and excessive production of these species can lead to sperm damage (reviewed in Aitken and Fisher, 1994; de Lamirande and Gagnon, 1999). Excessive ROS generation is a characteristic of morphologically abnormal spermatozoa and can lead to suppression of fertility. ROS induce lipid peroxidation of sperm plasma membranes (Storey, 1997) which decreases fluidity and increases permeability of the membranes (reviewed in de Lamirande and Gagnon, 1999), thus interfering with the ability of the sperm cell to take part in the membrane fusion events associated with fertilisation. Spermatozoa are particularly susceptible to this type of damage due to their high content of polyunsaturated fatty acids (de Lamirande and Gagnon, 1999). More recently, data has been published indicating that ROS also oxidise DNA (Kodama *et al.*, 1997; Shen *et al.*, 1997). In addition, available data indicates that H_2O_2

is the species responsible for motility loss in human (Aitken *et al.*, 1993b; de Lamirande and Gagnon, 1992a; de Lamirande and Gagnon, 1992b; Griveau *et al.*, 1995a), murine (Kodama *et al.*, 1995) and bovine (Blondin *et al.*, 1997) species. ROS treatment has been found to cause a rapid loss of intracellular ATP leading to a decrease in cAMP-dependent phosphorylation of axonemal proteins and consequently inhibition of motility (de Lamirande and Gagnon, 1992b; Leclerc *et al.*, 1996).

1.4.v. Hyperactivation

Sperm hyperactivation appears to be an essential event of capacitation. Sperm hyperactivation is associated with increased velocity, decreased linearity, increased amplitude of lateral head displacement and whiplash movements of the flagellum (de Lamirande and Gagnon, 1999). This type of motility usually takes place around the site of fertilisation and it is not a synchronous process in humans, as demonstrated by the fact that not all spermatozoa from an ejaculate display hyperactivated motility at the same time. Human spermatozoa also constantly switch from one pattern of hyperactivated motility to another, or from non-hyperactivated to hyperactivated motility (Burkman, 1990; de Lamirande and Gagnon, 1993b; Mortimer and Swan, 1995; Murad *et al.*, 1992).

The acquisition of hyperactivated motility is essential for penetration of the zona pellucida in addition to allowing detachment from the epithelium of the Fallopian tube. The concentration of bicarbonate required for mouse and hamster sperm hyperactivation (25 mM) was found to be much higher than the amount needed for the induction of capacitation (2.9 mM) (Boatman and Robbins, 1991; Neill and Olds-Clarke, 1987; Stauss *et al.*, 1995). Intracellular calcium has been shown to increase during hyperactivation of hamster spermatozoa from approximately 50 nM to 100-200 nM, however it was still lower than that observed during the acrosome reaction (300-400 nM) (Suarez and Dai, 1995). Moreover, the calcium increase was greater in the flagellum during hyperactivation and the head during the acrosome reaction (Suarez and Dai, 1995). Calcium is known to affect the curvature and the activity levels of the sperm axoneme (as reviewed in Lindemann and Kanous, 1989). Another possible target for calcium during hyperactivation is calcineurin, a major protein phosphatase in sperm (Kopf *et al.*, 1999) that is calcium-dependent and has been suggested to be involved in flagellar beat symmetry (Tash *et al.*, 1988).

Potential axonemal modifications may be related to phosphorylation of proteins, which is established to be involved in sperm motility (Leclerc *et al.*, 1996; Tash, 1990). Two proteins of 105 and 81 kDa were located to the fibrous sheath and were found to

be progressively phosphorylated on tyrosine residues when human spermatozoa were incubated under capacitating conditions (Leclerc *et al.*, 1996; Leclerc *et al.*, 1997; Tash, 1990). Furthermore, the significant increase in phosphorylation corresponded to the interval of time when human spermatozoa incubated *in vitro* acquire hyperactivated motility (Burkman, 1990).

It is evident that as with other capacitation events, timing of hyperactivation is crucial. Several studies have demonstrated that premature sperm hyperactivated motility can impede the passage of spermatozoa through the female reproductive tract (as reviewed in de Lamirande and Gagnon, 1999). It is therefore very important that this unique motility pattern occur within the vicinity of the oocyte. Also the conditions required for hyperactivation and capacitation are not always identical thus indicating that although hyperactivation is undoubtedly temporally associated with capacitation, the likelihood is that they are two separate processes.

1.5. Sperm-Zona binding

Once spermatozoa have penetrated the cumulus oophorus surrounding the oocyte, they come into contact with the zona pellucida. The zona pellucida is an acellular matrix that is secreted around the oocyte during folliculogenesis. In addition to forming a protective layer, it serves a species-specific function and provides the specific recognition site for spermatozoa for induction of the acrosome reaction. The zona pellucida also persists through the preimplantation stages of pregnancy.

The zona pellucida comprises three major glycoprotein species (ZP1-3), named in order of decreasing molecular weight. In the mouse these components are arranged in such a way that interconnecting filaments of heterodimers of ZP2 and ZP3 are held together by dimers of ZP1, to give an open porous matrix (Greve and Wassarman, 1985; Wassarman, 1988; Wasserman, 1990). These proteins are heavily glycosylated and possess both N-linked and O-linked oligosaccharide side chains that are linked to the polypeptide backbone by asparagine and serine/threonine residues, respectively. ZP3 is the most studied glycoprotein as it is thought to be the sperm receptor responsible for sperm/oocyte recognition and although there is great homology in this protein between species in relation to the amino acid sequence, they vary considerably in molecular mass presumably due to differences in glycosylation (Table 1.4.).

Species	Molecular weight of ZP3 (kDa)
Human	57-73
Mouse	83
Hamster	56

Table 1.4. Molecular weights of ZP3 glycoprotein in various mammalian species.

The O-linked carbohydrate side chains on ZP3 are believed to play a key role in the biological activity of this molecule, as demonstrated by the fact that when the oligosaccharides are removed and purified, they retain the properties of a sperm receptor (Wasserman, 1990). However, the purified oligosaccharides can lose their ability to induce the acrosome reaction, thus emphasising the importance of the polypeptide backbone in mediating this effect through the cross-linking of zona binding sites on the sperm surface (Leyton and Saling, 1989b; Wasserman, 1990).

Interestingly, ZP3 binds only to the plasma membrane overlying the acrosome in acrosome-intact sperm, suggesting that this region of the sperm plasma membrane possesses specific ZP3 binding proteins and/or receptors (Kopf *et al.*, 1999). When

the capacitated spermatozoon becomes loosely bound to the receptor ZP3, it is at this point the acrosome reaction is induced (specifically by this association with ZP3, the primary physiological inducer of the acrosome reaction).

1.6. Acrosome Reaction

Zona pellucida binding stimulates spermatozoa to undergo the acrosome reaction (Kopf and Gerton, 1991), which is a stimulus-secretion coupled exocytotic event in which the outer acrosomal membrane fuses with the overlying plasma membrane (reviewed in Yanagimachi, 1994). This causes a fenestrated structure over the surface of the acrosome, the purpose of which is to effect the release of the enzymatic components of the acrosomal vesicle (refer to Fig. 1.5.). The hydrolytic enzymes released from the acrosome digest the zona pellucida and the vigorous motility of the spermatozoa is then thought to drive the spermatozoa into the perivitelline space. As mentioned previously, only spermatozoa that bind to the zona pellucida and that have completed capacitation will undergo the acrosome reaction in response to ZP3.

Progesterone has also been proposed as another natural ligand for the acrosome reaction (Melendrez *et al.*, 1994; Meyers *et al.*, 1995; Osman *et al.*, 1989). It has been suggested that progesterone acts on a sperm $GABA_A$ receptor. It is also believed to work in synergy with the zona pellucida in the physiological acrosome reaction (Melendrez *et al.*, 1994; Roldan *et al.*, 1994). In the following section, factors thought to be involved in the acrosome reaction will be discussed in more detail (refer to Table 1.5. for summary).

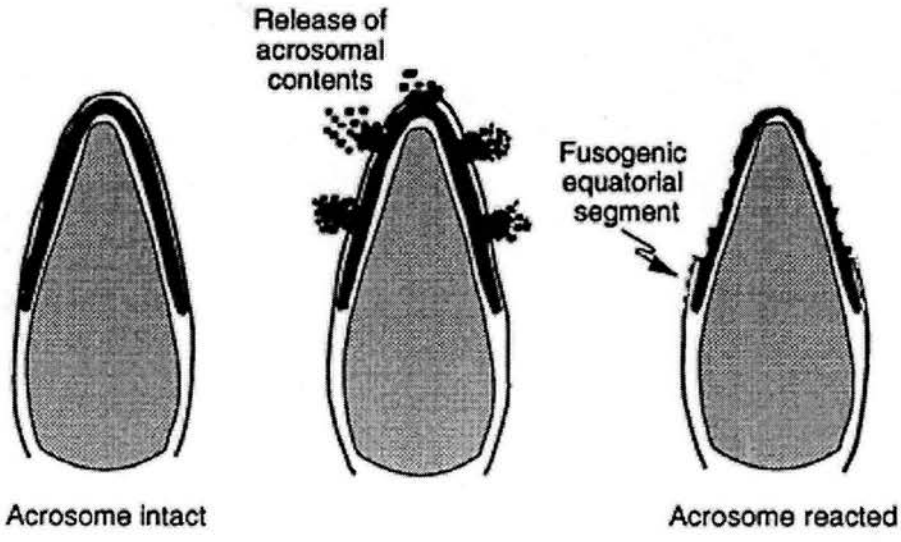


Figure 1.6. Schematic representation of the acrosome reaction. As a consequence of this process, enzymes are released in the acrosomal contents that facilitate zona penetration. A discrete band of plasma membrane around the equatorial region of the sperm head suddenly acquires the capacity to recognise and fuse with the oocyte. Taken from (Aitken, 1996b).

1.6.i. The biochemistry of the acrosome reaction: receptor tyrosine kinase

A 95 kDa receptor protein for ZP3 with the structure of a receptor tyrosine kinase was found in spermatozoa and it was suggested to activate a Na^+/H^+-exchanger which promotes cell alkalinization, membrane depolarisation and activation of a Ca^{2+} channel (Leyton and Saling, 1989a). A 95 kDa tyrosine phosphorylated protein with properties of hexokinase has also been identified in mouse spermatozoa (Kalab *et al.*, 1994). Progesterone has also been found to stimulate tyrosine phosphorylation of a 94 kDa human sperm protein which may be the 95 kDa receptor tyrosine kinase (Tesarik *et al.*, 1993b).

Epidermal growth factor (EGF) is found in high amounts in the female genital tract. Thus it is particularly interesting that a tyrosine kinase that is also a EGF receptor has been identified in the head of bovine spermatozoa and it is also thought to be involved in the acrosome reaction (Breitbart *et al.*, 1995; Lax *et al.*, 1994). During bovine sperm capacitation there is also an up-regulation in tyrosine phosphorylation of two proteins of 170 and 140 kDa, which could be the EGF receptor and phospholipase Cγ (PLCγ) respectively (Breitbart *et al.*, 1995). There is also a significant increase in PLCγ binding to the plasma membrane after its tyrosine phosphorylation (Spungin *et al.*, 1995).

Other receptors believed to play an important role in mammalian fertilisation include the protein PH20 which has been characterised in several species including the rat (Seaton *et al.*, 2000) and fox (ten Have *et al.*, 1998). It has been shown to migrate from the tail to the acrosomal domain during capacitation and endoproteolytic cleavage of PH20 in the rat serves to optimise its hyaluronidase activity immediately before fertilisation, thereby facilitating penetration of spermatozoa through the cumulus oophorus (Seaton *et al.*, 2000). Other binding partners for the zona pellucida proteins include galactosyltransferase, sp56 and spermadhesins which are thought to participate in the primary binding between sperm and zona pellucida and may initiate the exocytotic release of hydrolytic enzymes in the sperm head, culminating in the

acrosome reaction (McLeskey *et al.*, 1998). The proteins PH20, proacrosin, sp38 and sp17 are thought to participate in secondary binding between the acrosome reacted sperm and zona pellucida (McLeskey *et al.*, 1998).

Factor	Possible functions in acrosome reaction	Ca^{2+} dependence for activation	Involved in membrane fusion
Tyrosine kinases	• Transmembrane signalling • pY of PLCγ • Activation of a Na+/H+ exchanger • Activation of L-type Ca^{2+} channels	submicromolar	No
G-proteins	• Activation of adenylyl cyclase • Activation of $PLC\beta_1$ • H^+ efflux	submicromolar	No
Adenylyl cyclase/ cAMP/PKA	• Release of calcium from acrosomal stores	submicromolar	No
PKC	• Opening of plasma membrane calcium channels • Activation of PLA_2	micromolar	No
PLA_2/ arachidonic acid	• Enhancement of membrane fusibility • Ca^{2+} entry • Activation of PKC	micromolar	not known
PIP_2 specific PLC/IP_3	• Enhancement of membrane fusibility • Activation of PKC, PLA_2 • Actin depolymerisation • Release of Ca^{2+} from acrosomal stores	micromolar	yes
Actin severing proteins/actin depolymerisation	• Removal of the F-actin barrier to fusion	supramicromolar	yes

Table 1.5. Summary of some of the factors involved in the acrosome reaction (adapted from Breitbart and Spungin, 1997).

1.6.ii. The biochemistry of the acrosome reaction: G proteins

Pertussis toxin inhibits zona pellucida but not the ionophore-induced acrosome reaction (Endo *et al.*, 1987), thus indicating that G_i-like proteins are involved upstream to Ca^{2+} elevation. A model has been suggested in which sperm bind to the zona pellucida activating a cation channel causing a membrane depolarisation and a G_i-protein-dependent H^+ efflux, leading to intracellular alkalisation (as reviewed in Breitbart and Spungin, 1997).

1.6.iii. The biochemistry of the acrosome reaction: Adenylyl cyclase/cAMP/PKA

Intracellular cAMP levels are elevated during the acrosome reaction (Hyne and Garbers, 1979b; Ward and Kopf, 1993) and membrane bound adenylyl cyclase can be stimulated by the zona pellucida (Leclerc and Kopf, 1995). The function of these second messengers could be to open calcium channels, as detected in sea urchin spermatozoa (Cook and Babcock, 1993). Calcium release by a cAMP-dependent acrosomal channel in spermatozoa was found to be voltage dependent as it was inhibited by nifedipine (Spungin and Breitbart, 1996).

1.6.iv. The biochemistry of the acrosome reaction: Protein kinase C (PKC)/PLA_2/arachidonic acid

There are two possible role for PKC in the acrosome reaction: the first is to activate a plasma membrane calcium channel (Spungin and Breitbart, 1996) and the second is the activation of phospholipase A_2 (PLA_2). Stimulation of PLA_2 generates arachidonic acid which is metabolised to prostaglandins and leukotrienes by the enzymes referred to as cyclooxygenase (COX) and lipoxygenase (LOX) respectively. Prostaglandin production during the acrosome reaction is inhibited by the PKC inhibitor staurosporin and this inhibition can be overcome by the administration of exogenous arachidonic acid (Breitbart *et al.*, 1995).

Inhibitors of LOX and PLA_2 inhibit the acrosome reaction, whereby the products of these enzymes, arachidonic acid and 15-hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid (15-HETE) actually stimulate the acrosome reaction in capacitated spermatozoa (Breitbart *et al.*, 1995; Joyce *et al.*, 1987; Meizel and Turner, 1984). Exogenous PGE_2 enhances Ca^{2+} uptake (Shalev *et al.*, 1994) and stimulates the acrosome reaction which is completely inhibited by the LOX inhibitor NDGA, indicating that the LOX pathway is involved in the mechanism in which the COX pathway stimulates the acrosome

reaction (Breitbart *et al.*, 1995). It is thought that PGE_2 activates PLA_2 via PKC in a positive feedback loop, in order to release more arachidonic acid for 15-HETE synthesis which is crucial for the acrosome reaction (as reviewed in (Breitbart and Spungin, 1997)). It has been postulated that this feedback loop begins by the generation of diacylglycerol (from the hydrolysis of polyphosphoinositides by PLC) which activates PLA_2 directly during the acrosome reaction of ram spermatozoa (Roldan and Harrison, 1989). It is also possible that prostaglandins present in the seminal plasma cause the first activation of PLA_2. PGE_2 stimulates Ca^{2+} uptake by the cells, possibly via PKC, which would activate PLA_2 (Shalev *et al.*, 1994) and $PGF_{2\alpha}$ inhibits uptake of Ca^{2+} by bovine spermatozoa (Shalev *et al.*, 1994). These data are supported further by the fact that PGE_2, but not $PGF_{2\alpha}$ induced an increase in human sperm-oocyte fusion rates (Aitken and Kelly, 1985). The antagonism between these two prostaglandins could be a mechanism of regulation of Ca^{2+} entry into spermatozoa (Breitbart and Spungin, 1997).

1.6.v. The biochemistry of the acrosome reaction: Phosphatidyl-inositol bisphosphate (PIP_2)-specific PLC/inositol trisphosphate (IP_3)

Several roles have been proposed for the sperm membrane-bound PLC, one being that the diacylglycerol it produces would enhance membrane fusibility (Luk *et al.*, 1993; Siegel *et al.*, 1989), thus leading to activation of PKC and PLA_2. An additional action of PLC would be to hydrolyse phosphatidyl-inositol phosphate (PIP) and PIP_2 bound to actin severing proteins, consequently preventing the PIP_2 inhibition of these proteins (Goldschmidt-Clermont *et al.*, 1991; Janmey, 1994). It has also been demonstrated that progesterone induces diacylglycerol production in addition to inducing the acrosome reaction in human spermatozoa (O'Toole *et al.*, 1996).

1.6.vi. The biochemistry of the acrosome reaction: Actin-severing proteins/actin depolymerisation

The space between the plasma and outer acrosomal membranes is occupied by an F-actin network which is responsible for holding the PLCγ involved in the acrosome reaction at the membrane surface. In order for the acrosome reaction to take place it is crucial that dispersion of this network takes place, usually by actin depolymerisation. The fact that both actin depolymerisation (Spungin and Breitbart, 1996) and membrane fusion (Spungin *et al.*, 1995) require supramicromolar calcium indicates that the actin network constitutes the final barrier to fusion.

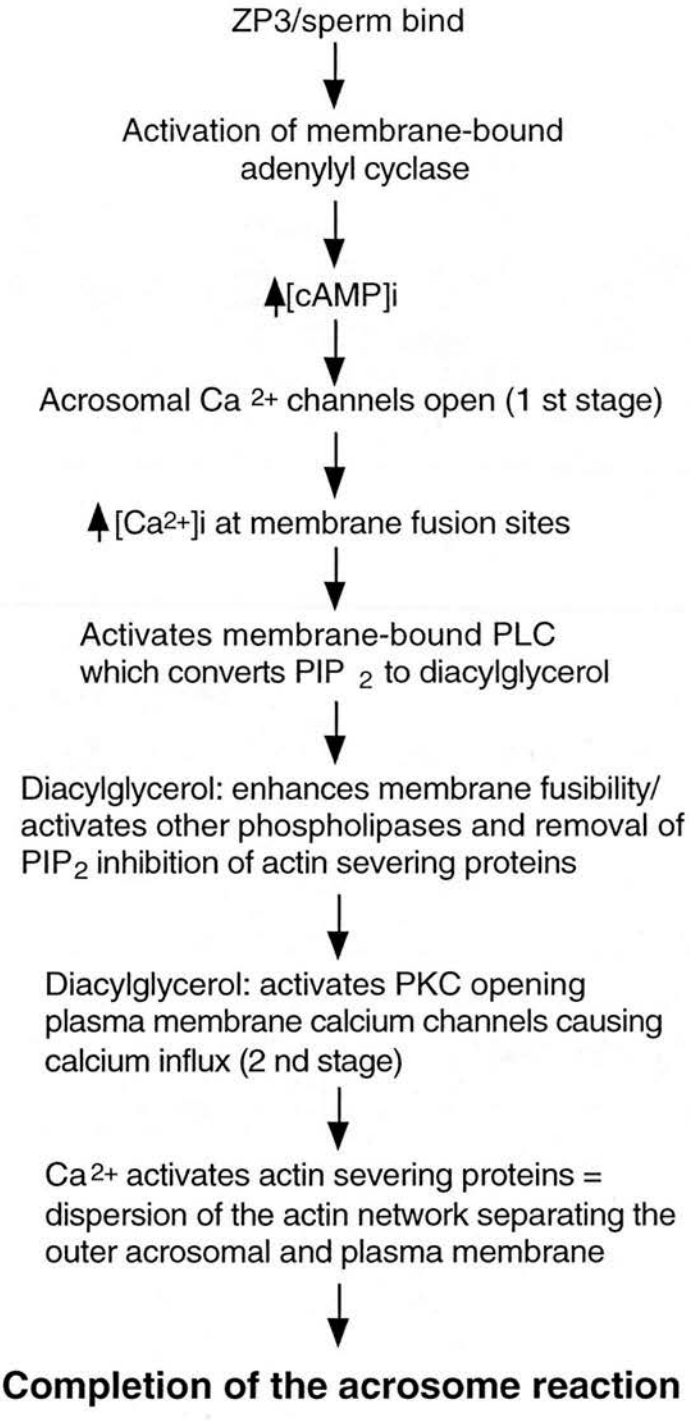


Figure 1.6. Flow diagram representing the main events taking place during the acrosome reaction.

1.7. Sperm-Oocyte fusion

Following the acrosome reaction, a discrete region of the plasma membrane around the equatorial segment acquires the ability to recognise and fuse with the vitelline membrane of the oocyte. However, this is unique to eutherian mammals, as in all other mammals it is the inner acrosomal membrane that fuses with the oolemma first (as reviewed in Yanagimachi, 1994). One of the visible indications of sperm-oocyte fusion is the sudden immobility of the sperm flagellum, which occurs seconds after fusion. This has been found to be the case for several species of animal including non-mammalian species (as reviewed in Yanagimachi, 1994). As a general rule, the entire tail is incorporated into the oocyte but it degenerates quite quickly after that.

The presence of complement factors in the equatorial region of the human sperm head have been postulated to play a role in the fusion process and these include CD46, C3 and C1q (Anderson *et al.*, 1993; Fusi *et al.*, 1991; Okabe *et al.*, 1990). Similarly, in the guinea pig a protein known as PH30 has been identified and it is thought to mediate sperm-oocyte fusion (Primakoff *et al.*, 1987).

Following sperm-oocyte fusion, the egg becomes activated by a protein referred to as oscillin, which is responsible for the induction of calcium transients leading to an elevation in the cytoplasmic concentration of free Ca^{2+} (Montag *et al.*, 1998; Parrington *et al.*, 1998; Parrington *et al.*, 1996). There is also a series of periodic membrane hyperpolarizations. What follows is a cascade of events such as the release of the cortical granules (to prevent polyspermy), the resumption of meiosis and the formation of the male and female pronuclei. At this point in time, the function of the spermatozoon is complete, in that it has successfully delivered its DNA to the oocyte and from then on embryogenesis can begin.

1.8. Closing Comments

This literature review has covered most of the current concepts related to how spermatozoa acquire their fertilising ability. It is evident that the whole maturation process is very complex and multifactorial in regard to its regulation, emphasising further the unique and specialised nature of these cells. Throughout the duration of these studies, the aim was to attempt to elucidate the external factors and intrinsic controls involved in epididymal maturation and the acquisition of fertilising ability in the rat. As mentioned previously, information regarding the cell biology of rat spermatozoa is limited. However, to gain an insight into the acquisition of full maturation status in these cells, would be of great value as it would allow the development of suitable assays for potential use as reproductive toxicological tools.

Chapter Two:

General Materials and Methods

Chapter 2 General Materials and Methods

This chapter details the general experimental techniques and procedures used throughout this study. Modifications of these methods are detailed in the appropriate chapters. All chemicals and reagents were obtained from various suppliers as stated in section 2.1.

2.1. Reagents

Biggers Whitten Whittingham Media (BWW) (Biggers *et al.*, 1971), was made up daily from the following ingredients: 90 mM NaCl (BDH, Poole, Dorset, UK), 4.5 mM KCl (BDH, Poole, Dorset, UK), 1.6 mM $CaCl_2$ (BDH, Poole, Dorset, UK), 1.1 mM $MgSO_4.7H_2O$ (BDH, Poole, Dorset, UK) 1.1 mM KH_2PO_4 (BDH, Poole, Dorset, UK), 25 mM $NaHCO_3$ (BDH, Poole, Dorset, UK), 5.6 mM glucose (Sigma, UK), 55 μM sodium pyruvate (Sigma, UK), 0.2% sodium lactate (Sigma, UK), 20000 IU penicillin/streptomycin (Calbiochem, Notts., UK) and 20 mM Hepes buffer (Gibco, Life Technologies, UK). The pH of normal BWW was 7.6 and was supplemented with 0.3% human albumin solution (Immuno Ltd, Kent, UK). Sperm diluting fluid (SDF) consisted of 50 g $NaHCO_3$ and 10 mls of formalin (BDH, Poole, Dorset, UK) made up to 1 litre with distilled water. The following reagents were obtained from Sigma, UK: N-tris[Hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS), luminol and lucigenin, both of which were dissolved in dimethyl sulfoxide (DMSO) which was from BDH, Poole, Dorset, UK. Horseradish peroxidase (HRP) was dissolved in BWW at a concentration of 2 mg/ml. Sodium dodecyl sulphate (SDS), phosphotyrosine, 3-Aminopropyltriethoxysilane (APES), Tween-20, Ponceau S concentrate, Trizma base (TRIS), Fast Red tablets, ß-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), ß-nicotinamide adenine dinucleotide, reduced form (NADH), N6, 2-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP), pentoxifylline (PTX), sodium orthovanadate, zinc chloride, phosphate buffered saline tablets (PBS) and lectin from Arachis Hypogea (AH). Complete™ mini protease inhibitor cocktail tablets and 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) were obtained from Boehringer Mannheim GmbH, Germany. Ammonium persulphate (AMPS), 2-mercaptoethanol, N, N, N'N'Tetra-methylethylenediamine (TEMED) and Silver Stain Plus kits were all from Biorad, Herts., UK. BDH also supplied the following reagents: acetone, sucrose, bromophenol

blue, Aquamount, haematoxylin and the ingredients for Scot's tap water; 2 g potassium bicarbonate and 20 g magnesium sulphate in 1 litre of distilled water. BCA protein assay reagent was obtained from Pierce, Chester, UK. Glycine (Calbiochem, Notts., UK), 40% acrylamide/bis-acrylamide stock solution (19:1) (Anachem, Leeds, UK), methanol and ethanol (Hayman Ltd, Essex, UK), nitrocellulose highbond super-C (Amersham, Bucks., UK), filter paper (Pharmacia Biotech, Herts., UK) and Seeblue molecular weight markers (Novex, Frankfurt, Germany). PY20 antiphosphotyrosine monoclonal antibody, ß-catenin monoclonal antibody, anti mouse IgG, HRP linked (raised in goat), anti-PKA (RI subunit used as an immunogen), anti-PKARIα and anti-PKARIIα monoclonal antibodies, calcineurin monoclonal antibody, anti AKAP 220 monoclonal antibody and Biomol® format A cyclic AMP enzyme immunoassay kit, acetylated version were all purchased from Affiniti, Exeter, UK. The HRP-conjugated monoclonal antibody against phosphotyrosine was obtained from UBI, New York, USA. ECL detection reagents and ECL Hyperfilm were from Amersham Life Sciences, Bucks., UK. Bovine serum albumin (BSA), Fura 2AM and Okadaic Acid were from Calbiochem, Notts., UK and rabbit anti mouse immunoglobulins (RAM) and anti-alkaline phosphatase (APAAP) were from Dako, Denmark. Eppendorfs were from Scotlab, Strathclyde, Scotland. Glass coverslips were from Chance Propper, West Midlands, UK. Improved Neubauer ruled 100 micron depth haemocytometers were obtained from BDH, Poole, Dorset, UK and Thoma ruled haemocytometers of 20 micron depth were from Weber, England. 12-well Hendley slides were obtained from Hendley Ltd, Essex, UK and PAP pens were from Bayer Diagnostics, Hampshire, UK. Petri dishes were purchased from Greiner Laboratechnik, Gloucestershire, UK. The vital stain ethidium homodimer 1 (EHD-1) was obtained from Molecular Probes, Cambridge, UK and diluted in DMSO to give a stock concentration of 10 mg/ml. Citifluor mountant was obtained from Citifluor Ltd, University of Kent, Canterbury.

2.2. Animals

The rats used in this study were of the Wistar Han (Charles River Laboratory, UK) variety as this is the chosen strain used in pre-clinical safety studies within GlaxoWellcome Research and Development. Animals used were within an age range of 12 and 24 weeks of age. This age range was defined to ensure that only sexually mature rats were used and also to provide an upper limit of maturity. The animals were maintained within the temperature range of 20-25°C and relative humidity was kept between 45 to 70%. The animals were fed with Rat and Mouse No. 1 Expanded Diet (Special Diets Services Ltd) and water from the domestic supply *ad libitum*.

The rats were killed by inhalation of a slowly rising concentration of CO_2 followed by dislocation of the cervical region in accordance with Schedule one of the Home Office Animal Act.

2.3. Extraction of Epididymal Spermatozoa

Although for human sperm there are basic World Health Organisation (WHO) guidelines (Organization, 1992) that may be used as a recommendation for certain aspects of analysis of these cells, such guidelines do not exist for other species. Indeed, the WHO manual (Organization, 1992) does not even cover all aspects of human sperm analysis. It is therefore up to individual laboratories to set their own experimental standards and conditions for the treatment of spermatozoa.

In species such as the mouse and rat, the sperm extraction procedure is completely different to that of the human and bull, as it is not possible to collect fresh ejaculated semen unless it is collected from the female following mating. However, this is not advisable as the female would have to be sacrificed due to the fact the male rat ejaculates into the uterus. In addition, the yield of sperm is very low when extracted in this way, consequently limiting the type and quantity of analysis. The most accessible way to obtain sperm from the rat or mouse is by epididymal extraction following the sacrifice of the animal. For this reason, all of the work in this thesis (unless otherwise specified) was carried out on epididymal spermatozoa.

The epididymides were removed from freshly killed animals by cutting into the lower abdominal cavity to expose the abdominal muscles and internal fascia. The fascia enclosing the inner cavity was cut and using fine forceps, the fat spilling from the open sac was pulled out revealing the testis and epididymis attached to it. This process was then repeated on the opposite side. Taking care to avoid the major blood vessels the epididymides were removed with fine scissors and laid on damp filter paper and

carefully trimmed of all fat. The organs were then separated into the caput, corpus and cauda (refer to Figure 2.1.). To limit contamination of the cells, blood was carefully expelled from each section of the epididymis by gently pressing forceps along the length of the divided organ towards the open cut ends of the vessels. The caput and cauda were then placed into separate petri dishes containing BWW (5 ml/ 1 caput or 1 cauda), prewarmed to 37°C and the corpus discarded when not required for use.

In order to evaluate the actual method of sperm extraction from the epididymis into media, two approaches were used using caudal epididymal sections from the same animal:

1. The caudal section was pierced with a needle and the sperm allowed to diffuse out into 5 ml of media for 15 minutes. The tissue was then removed from the petri dish.
2. To reduce epididymal debris contamination, the caudal section was gently pierced with a needle and squeezed rather than minced into 5 ml of BWW, using forceps. This was followed by removal of the epididymal tissue.

The petri dishes were immediately placed into an incubator at 37°C and left for a short time to allow the spermatozoa to disperse throughout the media.

The effectiveness of sperm retrieval by these two different techniques was evaluated by performing density counts as explained in Section 2.5. In addition motility assessments were carried out as described in Section 2.4, in order to determine the effect of these extraction methods on sperm viability.

The second approach was adopted as general procedure for sperm extraction because the yield of cells by this method was much greater, although it was not statistically significant (Refer to Fig. 2.2a.). In addition, although the percentage motile population of spermatozoa was slightly improved in the cells that had been allowed to diffuse into the media the effect was not significant (Fig. 2.2b). No obvious differences in the motion characteristics of the spermatozoa were observed.

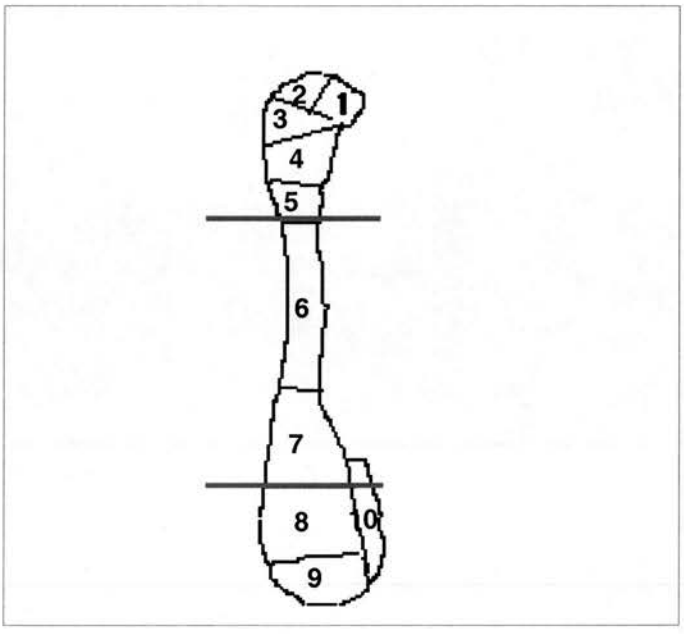


Figure 2.1. The rat epididymis is subdivided into ten arbitrary segments: 1-2 is the initial segment, 3-5 is the caput epididymis, 6-7 is the corpus epididymis and 8-10 is the cauda epididymis (adapted from Brooks, 1982). The purple lines indicate the cut-off points used for the division of the caput and cauda regions for all experiments detailed within this study.

2.3.i. The effect of temperature on rat spermatozoa

Conventionally, laboratories have attempted to mimic physiologic conditions, maintaining sperm suspensions at approximately 37°C throughout the collection, incubation and analysis phases. However, a consensus report compiled by several laboratories (Seed *et al.*, 1996) suggested that the acceptable temperature range is much broader, particularly towards the lower range. The working group stressed that it is more important to be consistent in the selection of temperature to avoid heat and cold shock (Seed *et al.*, 1996). They recommended that the tissue processing and sperm collection phases be conducted within the range of room temperature to 37°C and the analysis phase be conducted within the range of 34 to 37°C. In this study, spermatozoa were extracted from the freshly killed rat at a room temperature of 25°C and motility counts were also performed on prewarmed slides at this temperature. During incubation phases, spermatozoa were incubated at 37°C.

Niwa and Chang (Niwa and Chang, 1974a), in a study comparing various incubation conditions for the fertilisation of rat eggs *in vitro*, indicated that for IVF purposes, fertilisation rates were increased if the eggs and sperm were incubated with 5% CO_2 in air, when compared with air alone. It was postulated that this was due to the maintenance of a constant pH, as it was observed that the initial incubation media pH of 7.0-7.1, was elevated to a pH of 7.6-7.8 following 8-10 hours incubation in air without added CO_2 (Niwa and Chang, 1974a). In contrast, the pH of the media incubated in air with 5% CO_2, either changed slightly or not at all (Niwa and Chang, 1974a).

To determine the importance of supplementing air with 5% CO_2 for incubation purposes, rat sperm were incubated for 3 hours in nBWW with and without 3 mM PTX and 5 mM dbcAMP at 37°C in either air or air with 5% CO_2. The pH of the incubation media was taken at the beginning and end of the incubation period and motility counts were performed on the spermatozoa (Section 2.4.). No change in sperm motility, or the pH of the media was observed between those sperm incubated in air and air supplemented with 5% CO_2 (data not shown). Consequently, spermatozoa were incubated in air at 37°C, throughout this study. The diversity demonstrated between Niwa and Chang's (Niwa and Chang, 1974a) observations and our own, probably reflect the very different incubation conditions, not least the difference in incubation time. BWW is buffered with HEPES to a pH of 7.6 and evidently a short incubation time of three hours is not sufficiently long to potentiate any changes in media pH as a result of cell activity and lack of CO_2.

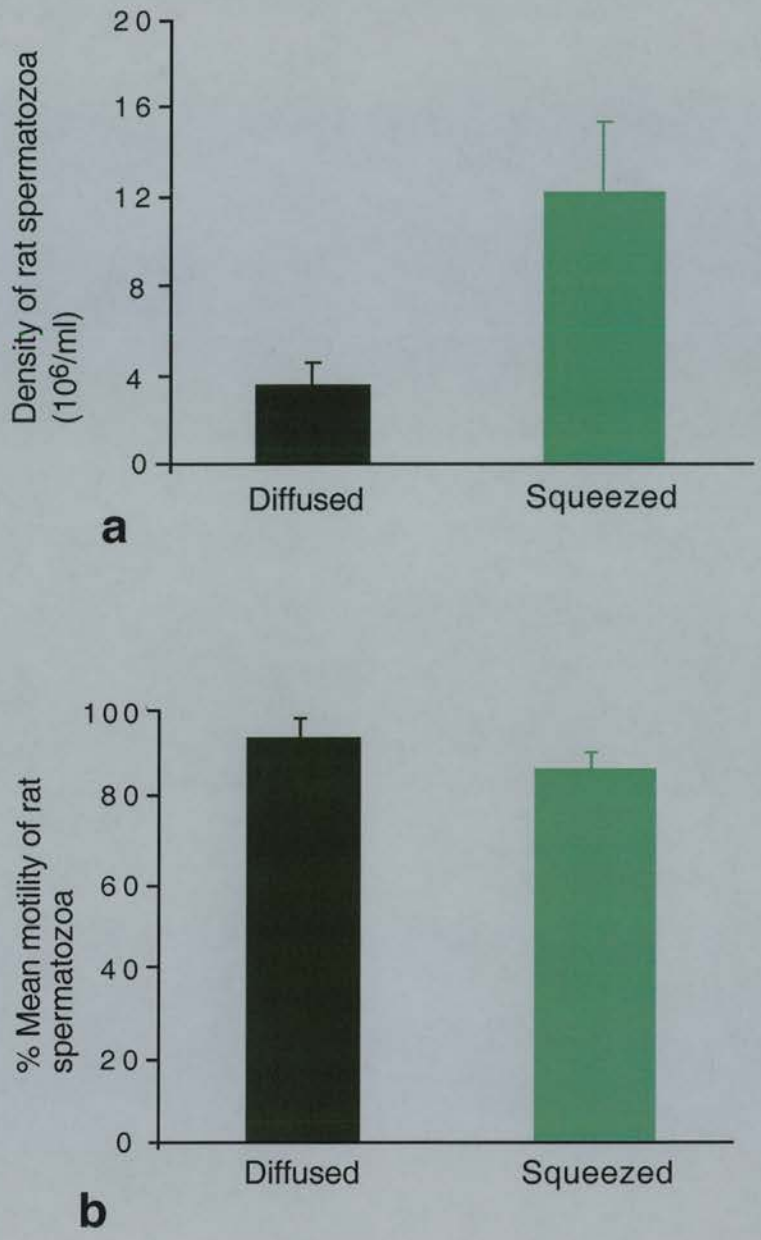


Figure 2.2. This figure represents the effect of different epididymal sperm extraction methods on the (a) density and (b) motility of retrieved caudal spermatozoa (n = 3).

2.4. Scoring Motility

10 μl of rat spermatozoa was pipetted onto a pre-warmed Thoma ruled haemocytometer of 20 micron depth. Using an eyepiece graticule with square grid, an area was defined and the motile and non-motile spermatozoa were counted in this same region. The field of vision was moved and the counting continued until a minimum of 100 sperm had been counted. The percentage of motile spermatozoa was calculated. In order to determine any immediately obvious effects of a treatment on the motility of the cells, motility counts were performed whenever possible following an incubation period.

2.4.i. The effect of slide depth on sperm motility

Rat spermatozoa are relatively large in comparison with other species (refer to Table 2.1. (Baccetti, 1984; Phillips, 1972)) and therefore it was of interest to determine if the chamber depth of a microscope slide had any effect on their motility. Motility counts were performed on caudal spermatozoa as described above, the only difference being the change in slide depth. Depths of 20, 50 and 100 micron slides were evaluated. As demonstrated in Fig. 2.3. there was no significant difference between the % motile population when the slide depths of 20 and 50 microns were compared. When 100 micron depth slides were used, the ability to count the % motile population was severely compromised due to the necessity of needing to examine several planes. Consequently, 20 micron slides were selected for use because they did not appear to impair the actual motile behaviour of the spermatozoa, as vigorous motile patterns could still be observed.

Species of spermatozoon	Length (μM)
Rabbit	46
Human	60
Mouse	120
Rat	190
Chinese hamster	250

Table 2.1. The above table represents the approximate average total length of a single spermatozoon from various mammalian species (Baccetti, 1984; Phillips, 1972).

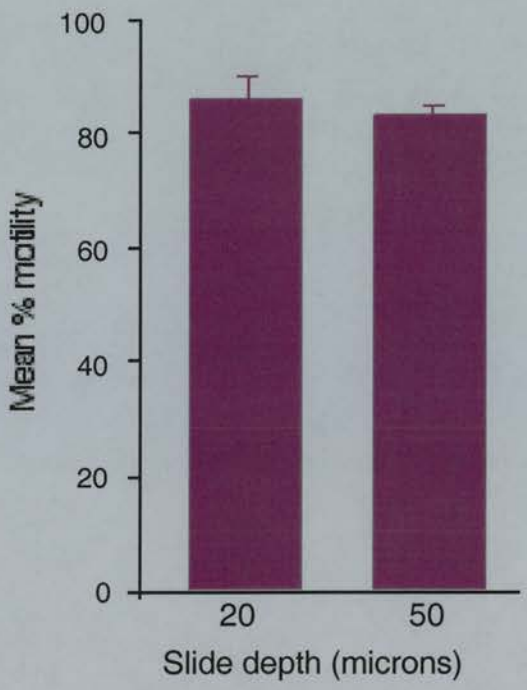


Figure 2.3. This figure represents the effect of slide chamber depth (microns) on the percentage population of motile caudal spermatozoa (n = 3).

2.5. Scoring Density

10 μl of rat spermatozoa was added to 190 μl of SDF to make up a 1 in 20 dilution. They were then mixed thoroughly using a Fisons Whirlimixer and 10 μl loaded onto an improved Neubauer haemocytometer counting chamber. They were left to settle for 5 minutes so that the sperm could sediment to a single depth of field. The number of spermatozoa in 5 large squares equalled 10^6/ml. All 25 large squares were counted and the number divided by 5 to give the concentration in 10^6/ml.

2.5.i. The effect of sperm concentration on sperm motility

The available literature indicates that no ideal concentration for the incubation of spermatozoa has been established, as working concentrations are widely variable from one laboratory to another. An obvious consideration when establishing such conditions is the purpose and length of time of the incubation period. Toyoda and Chang (Toyoda and Chang, 1974a) incubated rat sperm at a concentration of 3-6 x 10^6 sperm/ml media, although in another study they used 0.5-1.5 x 10^7 sperm/ml media (Toyoda and Chang, 1974b). In the hamster, epididymal spermatozoa were capacitated at concentrations of 16-27 x 10^6 cells/ml (Yanagimachi, 1969). Similarly, capacitation of mouse spermatozoa incubated at concentrations up to 20 x 10^6 cells/ml has also been demonstrated (Iwamatsu and Chang, 1970; Toyoda *et al.*, 1971). Shalgi *et al*, 1981 (Shalgi *et al.*, 1981) used rat spermatozoa at concentration ranges of 2 x 10^5 to 8 x 10^5 epididymal spermatozoa/ml media and 10^5 to 10^6 ejaculated spermatozoa/ml media for IVF purposes. Niwa and Chang (Niwa and Chang, 1974b) observed that rat spermatozoa incubated at high concentrations of 9.6-14.9 x 10^6 reduced their fertilising capacity following an incubation of 5-7.5 hours but this effect was not demonstrated after 4 hours incubation. They suggested that this may have been due to an exhaustion of nutrients.

To determine whether or not the concentration density exerted any effect on the activity of cells as measured by motility, rat spermatozoa obtained from the cauda epididymides of the same animal were diluted to 10^6/ml and 10 x 10^6/ml respectively and their motility was evaluated immediately and following 3 hours incubation at 37°C (Fig.2.4.).

For this investigative study, 10 x 10^6/ml seemed the most appropriate concentration for incubation periods as large numbers of cells were required in a relatively small volume. In addition, these conditions significantly promoted the activity of these cells in relation to the percentage motile population (refer to Fig. 2.4).

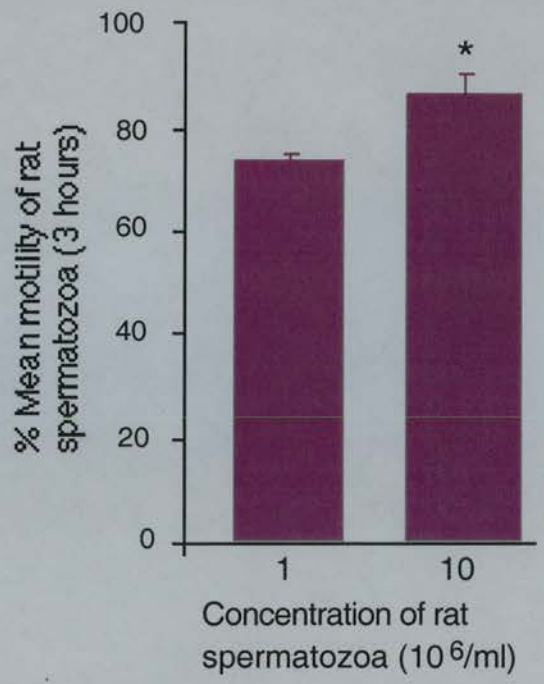


Figure 2.4. This figure represents the effect of different concentrations on the percentage population of motile caudal spermatozoa (n = 3).

2.6. Luminometry

Reactive oxygen species (ROS) were measured by luminol/lucigenin-dependent chemiluminescence using Berthold luminometers (LB9505, Berthold Analytical Instruments, Wildbad, Germany) at 37°C. Chemiluminescent probes such as luminol and lucigenin serve as substrates in oxygenation reactions as they emit increasing amounts of light in response to elevations in oxidant production (e.g., ROS produced by spermatozoa). The light is measured by a photon multiplier contained within the luminometer and therefore chemiluminescence is a continuous and quantitative method of measuring oxygenation activity or ROS. There are two sets of protocol used in this study, both of which employ the use of different reagents: **(i)** detects the presence of superoxide and **(ii)** detects the presence of hydrogen peroxide.

2.6.i. Detection of superoxide

Lucigenin is a charged compound used in the detection of superoxide. It is relatively cell impermeant and thus measures the production of superoxide extracellularly.

400 µl of the sperm suspension was pipetted into each plastic tube, taking care not to introduce any bubbles as this could provide an additional source of oxygen which may be detected during the course of the experiment. 4 µl of 25mM lucigenin was then added to each tube and then they were immediately placed in separate luminometer channels. The luminometer run was then started and it continued for a previously selected period of time. Increased superoxide production lead to increased fluorescence of lucigenin which was measured in counts per minute (cpm). It was possible to stop the run at any time so that reagents could be added.

2.6.ii. Detection of hydrogen peroxide

Luminol when used alongside HRP can measure the production of hydrogen peroxide intra- and extracellularly.

400 µl of sperm suspension was pipetted into each plastic tube as before and 8 µl of HRP followed by 4 µl 25 mM luminol was added to each tube. The tubes were placed into the channels and the run was started. The run was temporarily stopped to make further additions of reagents when required.

2.7. Extraction of sperm proteins

It has previously been demonstrated that different extraction conditions are required for rat caput and caudal spermatozoa (Chulavatnatol *et al.*, 1982), probably due to changes in the membrane and disulphide bonds during epididymal maturation. It was therefore of importance to establish conditions for this study that suited both types of cell.

The sperm protein extracting ability of four detergents was evaluated by SDS-PAGE (Poly-Acrylamide Gel Electrophoresis) (refer to Section 2.9.), followed by either Silver Staining of gels for the detection of proteins (Section 2.12.), or Wet Blot transfer of proteins to nitrocellulose and staining of the membrane with Ponceau S (Sections 2.10. and 2.11.):

2.7.i. OSGP (Octyl-β-D thioglucopyranoside)

OSGP is a nondenaturing nonionic detergent which is easily dialysed from solution and it is useful for solubilising membrane proteins.

On completion of an incubation period, motility counts were carried out on the spermatozoa. The sperm suspensions were washed three times with Tris washing buffer (8.3 ml of 0.375M Tris pH 6.8 diluted to 50 ml with distilled H_2O) at 500 g for 5 minutes. The supernatant was removed and 70 μl of 2% OSGP buffer was added to the pellet (70 μl/10 x 10^6 spermatozoa) and briefly vortexed. The OSGP buffer was made up as described in Table 2.2. and stored at -20°C until required for use. The sperm suspensions were incubated on ice for 60 minutes with occasional vortexing, followed by centrifugation for 20 minutes at 4480 g using a Howe centrifuge. The supernatant was retained from each tube and the pellet discarded. 5 μl of each supernatant was kept aside for use in a protein estimation assay. An equal volume of reduced sample buffer (refer to Table 2.3) was added to the remaining supernatant and boiled in a water bath at 100°C for five minutes. The purpose of the reduced sample buffer was to reduce the disulphide bonds binding the protein together and by boiling the samples it was possible to denature the proteins. Samples were allowed to cool and stored at -20°C until required for SDS-PAGE.

OSGP proved to be unsuitable for the purpose of this project as it did not extract caudal sperm proteins as efficiently as those from spermatozoa obtained from the caput epididymis as demonstrated by SDS-PAGE and Silver Staining (Fig. 2.5.).

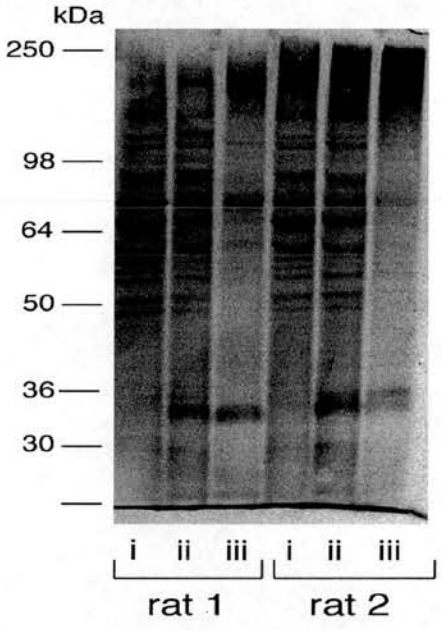


Figure 2.5. A Silver Stained gel exhibiting sperm proteins extracted with 2% OSGP. Spermatozoa were obtained from the **(i)** caput, **(ii)** corpus and **(iii)** cauda epididymis and incubated in BWW for 3 hours at 37°C prior to protein extraction. Proteins were separated by SDS-PAGE through 7.5% acrylamide gels and Silver stained as described in Section 2.12. (n = 3).

Reagent	Stock	Amount added
EDTA	50 mg/ml	250 µl
Leupeptin	1 mg/ml	250 µl
Pepstatin A	1 mg/ml	250 µl
Aprotinin	100 µg/ml	250 µl
E64	5 mg/ml	250 µl
Pefabloc	40 mg/ml	250 µl
Vanadate	100 mg/ml	250 µl
OSGP	5% w/v	10 ml
Tris pH 6.8	0.375 M	5 ml
Glycerol	100% v/v	2.5 ml
dH_2O	100% v/v	5.75 ml
Total volume of extract buffer (ml)		**25 ml**

Table 2.2. The above table represents the composition of OSGP extraction buffer.

Reduced Sample Buffer	Amount added
2-Mercaptomethanol (ml)	0.2
10% SDS (ml)	2.0
0.375 M Tris pH 6.8 (ml)	5.0
Sucrose (g)	1.0
Bromophenol Blue (mg)	0.5*
dH_2O (ml)	2.8
Total Volume (ml)	10

Table 2.3. The above table represents the composition of reduced sample buffer. *A 50 mg/ml solution of Bromophenol Blue in distilled water was added drop-wise until the colour was correct.

2.7.ii. CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate)

The zwitterionic detergent CHAPS is a sulfobetaine derivative of cholic acid. It is useful for membrane protein solubilisation when protein activity is important. It is useful over a wide range of pH (pH 2-12) and it is also compatible with the BCA Protein Assay.

Sperm suspensions were centrifuged at 700 g for 6 minutes at 25°C. The supernatant was removed and the samples were resuspended in approximately 2 ml (per 10 x 10^6 cells) of BWW without albumin (BWW-ALB). Albumin was excluded from BWW during this process to remove albumin. Following the second wash, the cells were resuspended at a concentration of 100 x 10^6/ml BWW-ALB containing protease inhibitors (1 Complete™ mini tablet per 10 ml of BWW-ALB) to prevent proteolysis. 10% CHAPS was added to the sperm suspension containing protease inhibitors at a 1 in 10 dilution, so that the final concentration of CHAPS in the sperm mixture was equal to 1%. The sperm suspensions were briefly mixed and then placed on a roller at room temperature for 15 minutes. Following the incubations, samples were centrifuged for 6 min at 1000 g, the supernatants retained and pellets discarded. 5 μl of each supernatant was kept aside for use in a protein estimation assay and the remainder was heated to 100°C for 5 min with an equal amount of reduced sample buffer (refer to Table 2.3.). Samples were then stored at -20°C until required for use.

CHAPS was used during this study to extract membrane proteins as Silver Stain analysis demonstrated that it was consistently the most efficient detergent when compared with Triton-X-100, in regards to the equal extraction of proteins from spermatozoa obtained from both the caput and cauda epididymis (Fig. 2.6.).

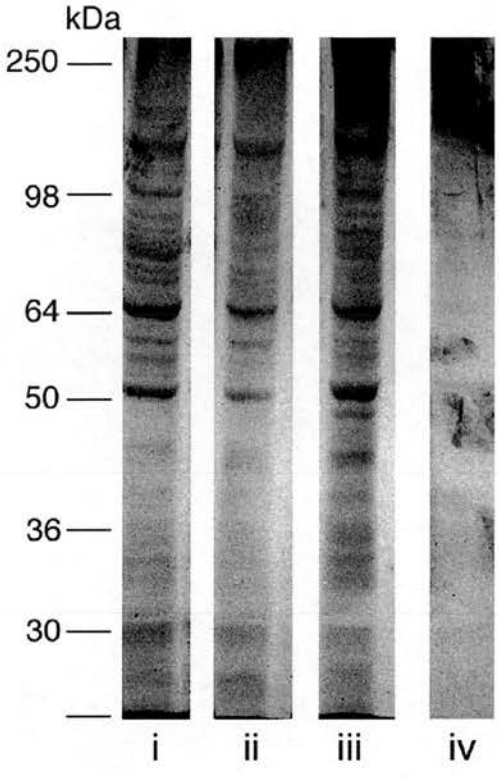


Figure 2.6. Silver Stained gel of sperm proteins extracted with 1% CHAPS, (i) and (ii) and 1% Triton-X-100, (iii) and (iv) respectively. Prior to extraction, rat spermatozoa obtained from both the caput and cauda epididymis were incubated for 3 hours in BWW at 37°C. Lanes (i) and (iii) represent caput sperm proteins and (ii) and (iv) are caudal proteins (n = 4).

2.7.iii. Triton-X-100

Triton-X-100 is suitable for solubilizing membrane proteins under non-denaturing conditions. Sperm protein extraction was performed as described for CHAPS except that Triton-X-100 replaced CHAPS. Triton-X-100 extracted proteins were stored at -20°C until required for use for analysis by SDS-PAGE and Silver Staining (Fig.2.6.).

2.7.iv. SDS (Sodium dodecyl sulphate)

SDS has a polar anionic sulfate group at one end of its structure and a straight chain non-polar region at the other end. The dual polar, non-polar nature of SDS allows it to solubilise many types of membrane proteins or polypeptides by imitating their structures. SDS also easily disperses most protein aggregates while causing denaturation of the proteins. It has been used in many applications where the maintenance of protein activity is not essential, such as gel electrophoresis.

The washing procedure for the sperm samples was the same as described in Section 2.7.ii. Following the washes in BWW-ALB, and resuspension of the spermatozoa in BWW-ALB containing protease inhibitors, 10% SDS was then added to the sperm suspension at a 1 in 10 dilution so that the final concentration of SDS was equal to 1%. Sperm suspensions were briefly mixed and the samples placed on a roller at room temperature for 1 hour. Following the incubations, samples were centrifuged for 6 min at 1000 g, the supernatants retained and the pellets discarded. 5 μl of each suspension was kept aside for use in a protein estimation assay and the remaining supernatant heated to 100°C for 5 min with an equal amount of reduced sample buffer (refer to Table 2.3). Samples were then stored at -20°C until required for use.

SDS was used throughout this study to extract proteins of the cytosol and fibrous sheath in addition to the membrane proteins, as Ponceau S staining demonstrated the efficiency of this detergent in the extraction of caput and caudal sperm proteins.

2.8. Protein Estimation Assay

In order to ensure equal loading of protein in each lane of a SDS gel for SDS-PAGE, protein estimations were carried out on every sample. All reagents used were part of a BCA protein assay kit obtained from Pierce, Chester, UK. Protein standards ranging from 50 μg to 250 μg of protein per ml were prepared and 45 μl of dH_2O was added to 5 μl of each protein extract. 1 ml of solution AB (100 parts solution A to 2 parts solution B) was added to 50 μl of each standard and protein extract dilution, in addition to the zero standard which consisted of 50 μl of dH_2O. The standards and extracts were then placed in a water bath heated to 60°C for between 15 and 30 minutes depending upon the intensity of colour change. A calibration curve using the standards was calculated and the protein concentration of the extracts measured at a wavelength of 562 nm using a Shimadzu UV-VIS scanning spectrophotometer UV-2101PC. The protein concentration of each extract was calculated and the volume of each extract to be loaded onto the gel, adjusted to equal the amount of protein found in the sample with the lowest concentration of protein. The minimum amount of protein required was set at 50 μg of protein per lane.

2.9. SDS-PAGE

SDS-PAGE is the most common method for separating proteins by molecular weight for immunoblotting purposes.

Following the assembly of the electrophoresis glass plates, gels were prepared in accordance with the guidelines set out in Table 2.4. TEMED and AMPS were only added to the mixture immediately before pouring, as they cause the gel to set. Approximately 3-6 ml of water saturated butanol was gently pipetted over the top of the resolving gel to prevent the gel setting with a meniscus, as this could affect the separation of the proteins in the outside lanes. Once the gel was set, the butanol was rinsed off with distilled water and the stacking gel poured over the top of the resolving gel. Combs were immediately placed in the stacking gel to form the wells for protein loading and great care was taken to eliminate bubbles in the mixture. The combs were removed from the set stacking gel and the newly formed wells were washed out with distilled water. The glass plate assembly containing the set gels was connected to the electrophoresis equipment ensuring that a tight seal was obtained and then electrode buffer (refer to Table 2.5. for composition) was poured into the chamber above the gels so that the wells were filled with the buffer. The appropriate volume of protein extract was gently pipetted into the bottom of the well to prevent mixing with the electrode

buffer and 15 μl of Seeblue marker was also loaded into at least one of the wells on each gel. The apparatus was then placed into an electrophoresis tank containing electrode buffer and bubbles were dispersed with a pipette to prevent interference with the distribution of the current. The apparatus was connected to a cold water supply to prevent overheating and the gels run at a constant current of 30 mA/ gel until the dye front reached the bottom of the glass plates.

Reagent	**% Acrylamide gel (40% 19:1 solution) / volume of reagent to be added (ml)**		
	3.6%	**5%**	**7.5%**
Acrylamide	2.7	10	15.1
0.375 M Tris Buffer pH 6.8	10	-	-
1 M Tris Buffer pH 8.85	-	30	30
10% SDS	0.3	0.8	0.8
Distilled Water	16.9	39.2	34.1
Temed	0.1	0.2	0.2
AMPS 10%	0.1	0.2	0.2
Total Volume	**30.1**	***80.4**	***80.4**

Table 2.3. The above table represents the composition of the mixtures required for the formulation of acrylamide gels. *80.4 ml is enough mixture for 2 large resolving gels of 1.5 mm thickness and the total volume in the 3.6% column will provide enough mixture for 2 stacking gels.

	Buffers				
	Electrode	**Wet Blot**	**1 M Tris pH 8.85**	**0.375 M Tris pH 6.8**	**10 x TBS (200 mM Tris, 1.5 M NaCl)**
Tris (g)	12.12	12.12	60.5	22.7	24.25
Glycine (g)	57.68	57.60	-	-	-
NaCl (g)	-	-	-	-	87.65
10% SDS (ml)	40	-	-	-	-
Methanol (ml)	-	800	-	-	-
dH_2O (ml)	3960	3200	500	500	1000
pH	-	-	8.85 (HCl)	6.8 (HCl)	7.6 (HCl)
Total volume (ml)	**4000**	**4000**	**500**	**500**	**1000**

Table 2.4. The above table details the composition of the basic buffers required for SDS-PAGE and Western Blot analysis. 4 litres of electrode buffer was sufficient for 1 gel tank (2 gels) and 4 litres of Wet Blot buffer filled 1 blot tank and also provided an additional amount for soaking of the membranes, filter paper and Scotchbright pads. Buffers were adjusted to a specific pH with concentrated HCl using an electronic pH meter.

2.10. Wet Blot Transfer

Taking care to wear latex gloves, 8 pieces of filter paper and 1 piece of nitrocellulose were cut to the size of the resolving gel, without the stacking gel (approximately 11.5 cm x 16.5 cm). Small cuts were made on one side of the nitrocellulose for identification and orientation purposes and together with the filter paper they were soaked in Wet Blot Transfer buffer (refer to Table 2.5.) for a minimum of 15 minutes. The Scotchbright pads were briefly soaked in Wet Blot Transfer buffer and then one pad laid on the inside of the open cassette. The pad was covered with 4 sheets of the pre-soaked filter paper and special care was taken to remove air bubbles by rolling over the filter paper with a sterile pipette to ensure good contact between the gel and the membrane. The soaked sheet of nitrocellulose was placed on top of the filter paper. The stacking gel was discarded and the resolving gel removed from the glass plates and placed on top of the membrane. The remaining 4 sheets of filter paper were placed on top of the gel and once again bubbles were removed. The second soaked Scotchbright pad was placed on top, the cassette closed tightly and placed in the tank filled with Wet Blot Transfer buffer. It was important to place the cassette into the tank in the correct orientation i.e. the gel should be nearest to the negative electrode and the nitrocellulose closest to the positive electrode so that the proteins migrate from the negative to the positive (gel to nitrocellulose ~ refer to Figure 2.7.). The cooling coil was connected to the cold water supply, the lid was closed and the apparatus run at a constant voltage of 25 V overnight (25 V/tank).

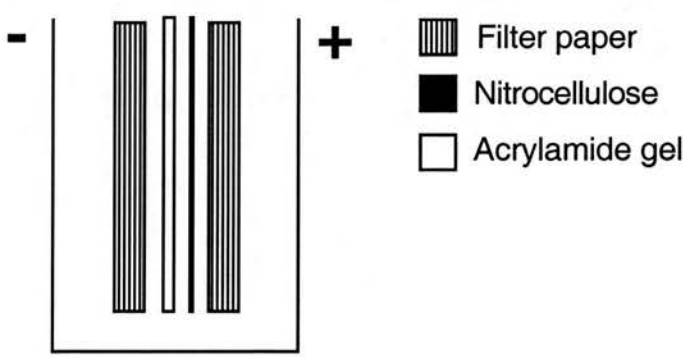


Figure 2.7. Diagrammatic representation of the gel apparatus in relation to the positive and negative electrodes.

2.11. Western Blot

Following Wet Blot Transfer of proteins, the gel and filter papers were discarded and the nitrocellulose was placed into a clean plastic tray. Ponceau S was used routinely to verify transfer of the proteins in each lane as the staining is rapid but not permanent and the red stain can be easily washed away. A stock solution of Ponceau S was prepared according to the manufacturer's guidelines; 20 ml diluted with 180 ml of deionized distilled water to give a final concentration of 0.2%. The nitrocellulose sheet was briefly washed once in Ponceau S stock solution and then fresh solution was added for 5 minutes with gentle agitation. The Ponceau S was poured off and the nitrocellulose washed several times with deionised distilled water until the red stain of proteins became clear. The nitrocellulose was subjected to further washes with fresh deionised distilled water to remove further traces of the stain.

10 x Tris buffered saline (10 x TBS, refer to Table 2.5.) was diluted 1 in 10 to give 1 x TBS. 3 g of bovine serum albumin (BSA) was dissolved in 100 ml of 1 x TBS to give a 3% BSA solution and the membrane was incubated in this for 1 hour at room temperature (50 ml/ gel) on a rocker to block non-specific binding sites.

The membrane was briefly rinsed with deionized distilled water and probed with PY20 (1 mg protein/ml), a monoclonal antibody against phosphotyrosine at a 1 in 1000 dilution (20 μg protein per membrane) in 1 x TBS 0.1% Tween-20-1% BSA for 2 hours at room temperature. Tween-20 is a non-denaturing, non-ionic detergent that is used to prevent non-specific binding to hydrophobic materials. This was followed by repeated washes in 1 x TBS 0.1% Tween-20 (1 x 15 min and 3 x 5 min) and incubation with anti mouse IgG, HRP linked (raised in goat), at a concentration of 1 in 6000 in 1 x TBS 0.1% Tween-20-1% BSA for 1 hour. This incubation was followed by further washes in 1 x TBS 0.1% Tween-20; 1 x 15 min and 3 x 5 min. An ECL kit was used for detection of the proteins; 10 ml of Solution A and 10 ml of Solution B were mixed together, poured onto the nitrocellulose followed by agitation for 1 minute. The nitrocellulose was gently shaken dry, wrapped in clear film and secured in a cassette. In the dark room, ECL film was placed over the nitrocellulose, the cassette was closed tightly and the film exposed for varying lengths of time. The film was gently agitated in developing solution until protein bands became clear, then rinsed with tap water and placed in fixative solution until the film became clear. Following further washes in tap water, the film was allowed to dry and photographed.

2.11.i. Controls

Following separation of proteins by SDS-PAGE through 7.5% acrylamide gels and Wet Blot transfer of proteins onto nitrocellulose, the specificity of antibodies for Western Blot analysis was determined by the following procedures:

1. Exclusion of the primary antibody during Western Blot analysis.
2. Preabsorption of the primary antibody with either phosphotyrosine or tyrosine for 1 hour at room temperature to determine antibody specificity for phosphotyrosine.
3. Replacement of the phosphotyrosine antibody with an alternative primary antibody against an antigen unlikely to be present on rat spermatozoa; for example β–catenin and oestrogen receptor. Alternative antibodies were added to the membrane at the same concentration as the anti-phosphotyrosine antibody.

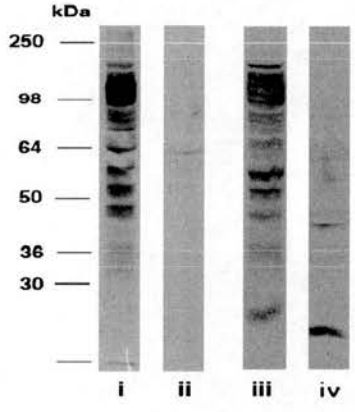
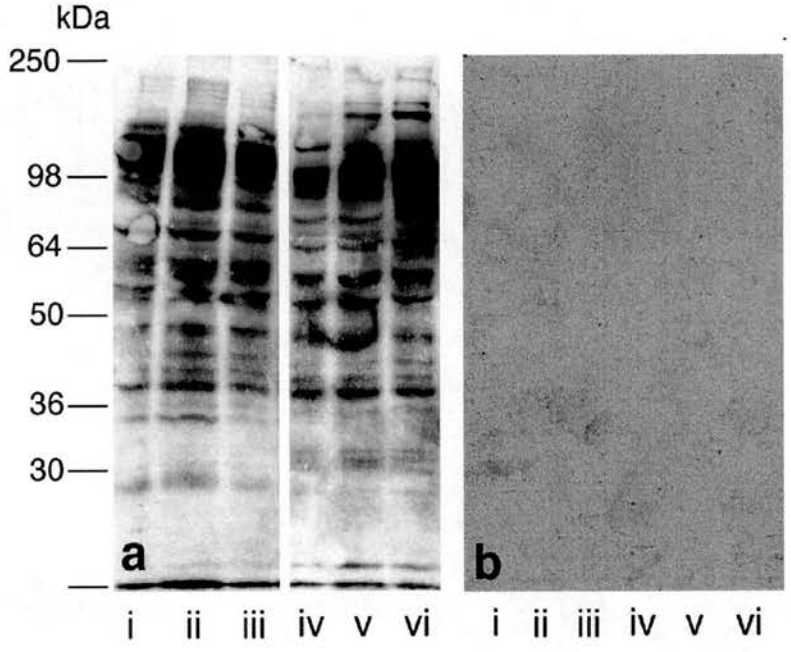


Figure 2.8. Western Blot analysis of tyrosine phosphorylated sperm proteins. The proteins were extracted with either 1% CHAPS **(i)** and **(ii)**, or 1% SDS **(iii)** and **(iv)**, from rat caput spermatozoa that had been incubated for three hours at 37°C. During Western Blot analysis lanes **(i)** and **(iii)** were probed with both the primary antibody, PY20 and the secondary antibody, anti mouse IgG, HRP linked (raised in goat). Lanes **(ii)** and **(iv)** were probed with the secondary antibody only (n = >6).

Figure 2.9. Western Blots of sperm tyrosine phosphorylated proteins. **(a)** and **(b)**: Caput (**i** to **iii**) and caudal (**iv** to **vi**) spermatozoa were incubated for 3 hours at 37°C in BWW with the following treatments prior to extraction with 1% SDS: **(i)** and **(iv)**, BWW; **(ii)** and **(v)**, 3 mM PTX; **(iii)** and **(vi)**, 3 mM PTX + 2 mM NADPH. Figure **(a)** represents the proteins probed with PY20 and anti mouse IgG, HRP linked (raised in goat) and **(b)** represents duplicates of the same samples probed with the same antibodies except that the PY20 had been preabsorbed with 20 mM phosphotyrosine for 1 hour at 25°C (n = >6). **(c)** Western Blot of 1% CHAPS extracted caput sperm proteins following incubation in BWW for 3 hours at 37°C. The proteins were probed with HRP-conjugated monoclonal antibody against phosphotyrosine that had previously been preabsorbed with different concentrations of phosphotyrosine or tyrosine for 1 hour at 25°C. **(i)** 10 mM phosphotyrosine; **(ii)** 1 mM phosphotyrosine; **(iii)** 100 μM phosphotyrosine; **(iv)** 10 mM tyrosine; **(v)** 1 mM tyrosine; **(vi)** 100 μM tyrosine; **(vii)** antibody only; **(viii)** no antibody (n = 2).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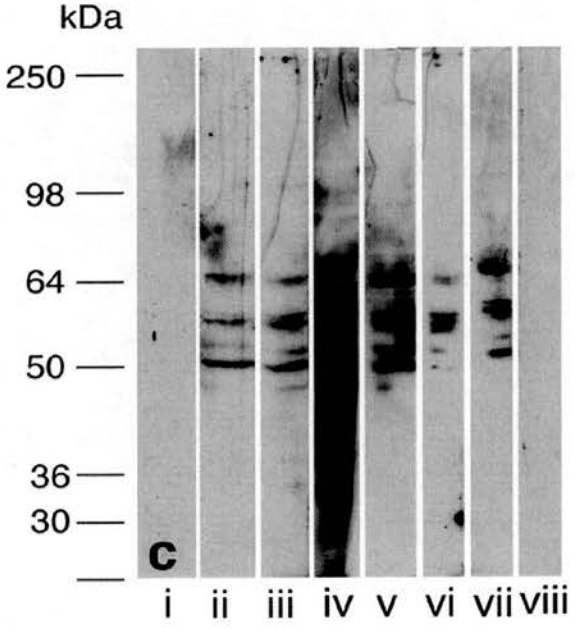
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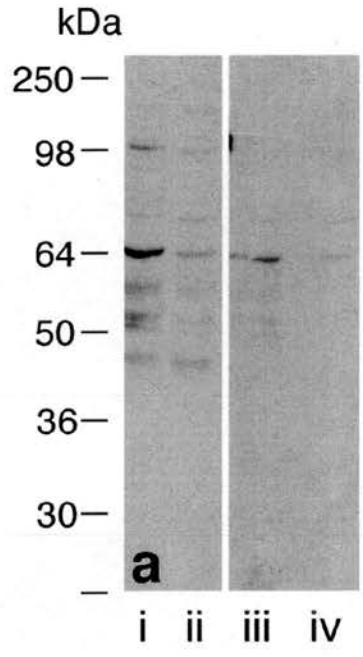
Figure 2.10. **(a)** Western blot of sperm proteins extracted with 1% CHAPS following incubation for 3 hours in BWW at 37°C. **(i)** and **(ii)** represent proteins extracted from caput and caudal spermatozoa respectively, probed with PY20 and anti mouse IgG, HRP linked (raised in goat). **(iii)** and **(iv)** represent the duplicate samples of **(i)** and **(ii)** except that the primary antibody PY20, was replaced with an antibody against β-catenin (n = 1). **(b)** Western blot of sperm proteins extracted with 1% SDS. **(i)** and **(iii)** represent caput sperm proteins from the same animal, following incubation for 3 hours in BWW at 37°C. while **(ii)** and **(iv)** are sperm proteins extracted from caudal cells of the same animal following stimulation with 3 mM PTX and 5 mM dbcAMP for three hours in BWW at 37°C. **(i)** and **(ii)** were probed with PY20 and anti mouse IgG, HRP linked (raised in goat) and for Western Blot analysis of **(iii)** and **(iv)**, the primary antibody PY20 was replaced with a monoclonal antibody against the E_2 receptor (kindly donated by Richard Sharpe's laboratory) (n = 2).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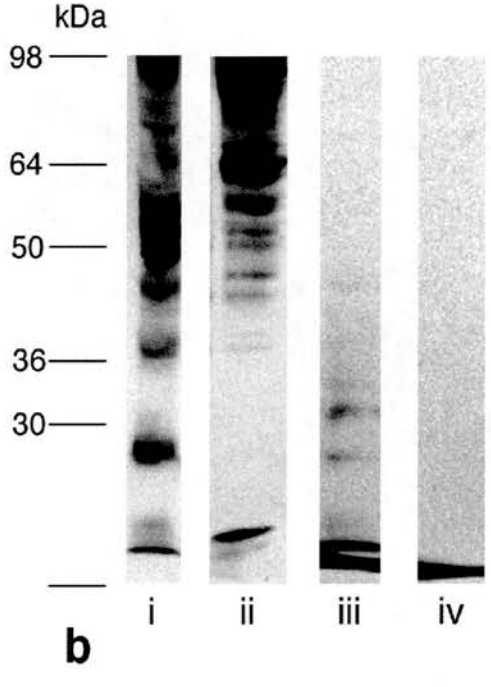
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2.12. Silver Staining of proteins

Silver Staining of proteins in gels was carried out using the Silver Stain Plus kit from Biorad, UK. Following gel electrophoresis, gels were placed in the Fixative Enhancer Solution which consisted of the following ingredients: 400 ml of reagent grade methanol (50% v/v), 80 ml of reagent grade acetic acid (10% v/v), 80 ml of Fixative Enhancer Concentrate (10% v/v) and 240 ml of deionized distilled water (30%v/v) to give a total volume of 800 ml which was an adequate volume for a large gel. The gel was gently agitated in the fixative for 30 minutes.

The Fixative Enhancer Solution was decanted from the staining vessel and gels were rinsed in 800 ml of deionised distilled water for 20 minutes with gentle agitation. The water was discarded and replaced with fresh rinse water for an additional 20 minutes.

The staining solution was prepared within 5 minutes of use as follows: 105 ml of deionised distilled water was placed into a large flask and stirred with a Teflon coated stirring bar and then 15 ml of Silver Complex Solution was added followed by 15 ml of Reduction Moderator Solution and 15 ml of Image Development Reagent respectively. Immediately before use, 150 ml of Development Accelerator Solution that had previously been warmed to room temperature was added to the beaker and stirred well. The contents of the beaker were then added to the staining vessel and the gel was stained with gentle agitation. Once the desired stain was reached gels were placed in 400 ml of 5% acetic acid to stop the reaction, for a minimum of 15 minutes. Gels were rinsed in high purity water for 5 minutes and then photographed.

2.13. Procedure for cleaning slides

Hendley slides were placed into a metal slide rack and washed in 70% ethanol in a large staining dish for 5 minutes on a rocker. They were then briefly dipped in deionized distilled water and allowed to dry in a fume hood. The procedure was repeated and the slides stored in a box with a tight fitting lid until required for further use.

2.14. Immunocytochemistry

Following various treatments, 50 μl of each sperm suspension was added to 150 μl of PBS to give a concentration of 2.5 x 10^6/ml. A PAP pen was used to outline each dot of a Hendley slide to prevent the spread of solutions. 15 μl of the sperm in PBS was then pipetted on to each well of a 12 well Hendley slide and left to dry overnight in a fume hood. Slides were either fixed in 1% paraformaldehyde for 10 minutes or 100% methanol and 3% hydrogen peroxide for 30 seconds. The slides were washed twice in distilled water for 2 mins each followed by a wash in 1 x TBS 0.1% Tween-20 for 20 mins. 20 μl of PY20 (diluted 1 in 10 in 1 x TBS to give a final concentration of 0.1 mg/ml) was pipetted on to each well and incubated for 1 hour in a humid box. Following the incubation, slides were washed twice for 5 min each in TBS Tween-20 0.1% and incubated with RAM, diluted 1 in 25 with a 1 in 10 solution of normal rabbit serum in 1 x TBS for 30 min. The washes were repeated (2 x 5 min) and the slides incubated for 1 hour with APAAP, diluted 1 in 50 in 1 x TBS. Following further washes in 1 x TBS 0.1% Tween-20 (2 x 5 min) the incubations with RAM and APAAP were repeated but for a reduced duration of 15 min each, with 2 x 5 minute washes in between. The wash step was repeated (2 x 5 min) and 20 μl of Fast Red substrate was added to each well and incubated for 18 min. The slides were washed in 1 x TBS 0.1% Tween-20 for 5 min followed by a wash in gently running tap water. They were then dipped in haematoxylin for 45 seconds followed by tap water, Scots water for approximately 30 seconds and tap water again. Staining of the cells was checked under bright field microscopy prior to mounting in Aquamount, followed by drying overnight. The slides were observed using an Olympus AX70 microscope and a minimum of 200 cells on each slide were counted for staining. For the control slides, PY20 was replaced with a monoclonal antibody against ß-catenin, or substituted with normal mouse serum at an equal concentration of protein to the antibody itself (0.1 mg/ml).

2.15. Measurement of intracellular cAMP generation in spermatozoa

Cyclic AMP was extracted from cells by centrifugation at 600 g for 6 minutes at room temperature to remove the BWW, followed by incubation with 0.1 M HCl (250 μl/10 x 10^6 cells) for 10 min at 25°C. One ml of sperm at a concentration of 10 x 10^6/ml was an adequate amount for 2 wells i.e. a sample and one duplicate. The samples were centrifuged once more at 600 g for 6 minutes at room temperature and the supernatants retained for use in the assay. Intracellular cAMP concentrations of spermatozoa incubated with various treatments were measured using the Biomol® format A cyclic AMP enzyme immunoassay kit, acetylated version (Biomol®, PA, USA), adapted from the manufacturers guidelines.

All of the reagents used were from the Biomol® kit and they were allowed to adjust to room temperature for 30 minutes prior to opening. The standards and other reagents were prepared as follows shortly before required for use:

500 pmol/ml = 990 μl 0.1M HCl + 10 μl of cAMP standard solution.
50 pmol/ml = 900 μl 0.1M HCl + 100 μl of 500 pmol/ml standard.
25 pmol/ml = 500 μl 0.1M HCl + 500 μl of 50 pmol/ml standard.
2.5 pmol/ml = 900 μl 0.1M HCl + 100 μl of 25 pmol/ml standard.
0.5 pmol/ml = 800 μl 0.1M HCl + 200 μl of 2.5 pmol/ml standard.
0.05 pmol/ml = 900 μl 0.1M HCl + 100 μl of 0.5 pmol/ml standard.
0.025 pmol/ml = 500 μl 0.1M HCl + 500 μl of 0.05 pmol/ml standard.
0.005 pmol/ml = 800 μl 0.1M HCl + 200 μl of 0.025 pmol/ml standard.

The zero/ non-specific bound (0/NSB) standard consisted of 1 ml 0.1M HCl. The acetylation reagent was made up by adding 0.5 ml acetic anhydride to 1 ml of triethylamine and the washing buffer consisted of 10 ml of wash concentrate made up to 100 ml with deionised water.

The pipette tips were pre-rinsed with the reagent and fresh tips were used for each sample. 10 μl of acetylation reagent for each 200 μl of standard or sample (eg. 12.5 μl/ 250 μl of sample) was added to each tube and vortexed for 2 seconds. Using the provided assay layout sheet, the number of wells to be used was determined and any spare wells were replaced with the dessicant back into the Ziploc pouch and stored at 4°C until required for further use. 50 μl of pink neutralising reagent was pipetted into each well except for the total activity (TA) and blank wells. 100 μl of the 0/NSB tube was pipetted into the 0/NSB and the Bo (0 pmol/ml standard) wells. As a rule standards and samples were pipetted to the bottom of the wells and reagents were

added to the side of the well to avoid contamination. 100 μl of each standard and sample was pipetted into the appropriate wells including a duplicate for each and 50 μl of 0.1 M HCl was added to the 0/NSB wells. 50 μl of yellow antibody solution was added to each well except for the blank, TA and 0/NSB wells. At this point the 0/NSB wells were purple, the blank and TA were empty and therefore colourless and all the other wells were brown. The plate was covered with the plate sealer and incubated at room temperature for 2 hours on a plate shaker at 500 rpm.

Following the 2 hour incubation, the wells were emptied and washed three times with 200 μl of wash solution in each well. The wells were then tapped dry on lint free paper and 5 μl of blue conjugate was added to the TA wells followed by 200 μl of p-Npp substrate solution to every well. The plate was incubated at 25°C for one hour without shaking. To stop the reaction 50 μl of stop solution was added to each well and the plate was read immediately using a Labsystems Multiskan® MCC/340 plate reader. The optical density was read at 405 nm and as the plate reader was not able to be blanked against the blank wells, the mean optical density of the blank wells was manually subtracted from all of the readings.

The Assay zap programme from Biosoft, Cambridge, UK was used to calculate the intracellular cAMP concentration of the samples.

2.16 Measurement of intracellular Ca^{2+}in spermatozoa

Intracellular Ca^{2+} levels in rat spermatozoa were evaluated using a Shimadzu RF-5301PC Intracellular Ion Measurement System with Super Ion Probe software. Sperm at a concentration of 10 x 10^6/ml were incubated with 4 μM Fura 2AM for 30 minutes at 37°C and protected from light. This was followed by centrifugation of the sperm samples at 700 g for six minutes, the supernatant was removed and the pellets were resuspended in BWW. To obtain the Rmax value, 5% Triton X was added to the sperm followed by 10 mM EGTA to give the Rmin value. Duplicate sperm samples were also set up that had not been incubated with Fura 2AM in order to establish background fluorescence. Intracellular calcium levels were calculated in accordance with the spectrofluorimeter manufacturer's guidelines.

2.17 Procedure for TESPA coating of slides

Hendley slides previously cleaned in 70% ethanol (see earlier) were immersed in 2% v/v APES in acetone for 30 seconds. 6 ml of APES in 300 ml of acetone was a

sufficient amount to coat a rack of slides in a large staining dish. The slides were allowed to dry in a fume hood and the process repeated. Once the slides were dried for the second time they were immersed in acetone for 20 seconds followed by immersion in deionised distilled water for a further 20 seconds. They were allowed to air dry overnight and stored in a box with a tightly fitted lid until required for use. Slides were coated with APES for acrosome reaction test assays to help maintain a high concentration of cells attached to the slide.

2.18 Acrosome Reaction Test Assay

To examine the viability of non-fixed sperm, EHD-1 was used as it is impermeant and has a high affinity for DNA. Additionally it was able to withstand the multiple washing and cell permeation procedures necessary for staining by Arachis Hypogea (AH); Cooper and Yeung, 1998 (Cooper and Yeung, 1998) found that because sperm have to be permeabilised to admit the lectin, some vital dyes were lost due to the membrane damage thus resulting in reduced estimates of the % of nonviable cells. This was the case for propidium iodide and my own studies confirmed their findings.

Immediately prior to use, the 10 mg/ml DMSO stock solution of EHD-1 was diluted further in PBS to give a working solution of 1 mg/ml. An appropriate amount was added to the sperm suspension to give a final concentration of 1 μg/ml and gently mixed for one minute at room temperature protected from light. This was followed by the addition of salmon sperm DNA at a final concentration of 525 μg/ ml sperm for a further two minutes under the same conditions. The aim of this step was to bind excess EHD-1, because if there is surplus dye remaining when the cells are permeabilised with methanol all of the cells would stain positive, thus eliminating the ability to identify initially viable cells from the dead. The samples were centrifuged at 500 g for 6 minutes at 25°C, the supernatant discarded and the pellet resuspended in PBS/BSA (4 mg BSA/ml PBS). The samples were centrifuged once more under the same conditions and then resuspended in ice cold methanol (250 μl/10 x 10^6 sperm) for 30 seconds. This was followed by centrifugation at 2000 g for 30 seconds. The sperm pellet was then resuspended in 1mg/ml AH (100 μl/10 x 10^6 sperm) for 15 minutes protected from the light. The sperm suspensions were washed twice in PBS/BSA at 2000 g for 1 minute at 25°C and resuspended in PBS (250 μl/10 x 10^6 sperm). 10 μl of the mixture was spread onto each dot of a 12 well APES coated Hendley slide and allowed to dry. The slides were mounted with Citifluor and viewed under an Olympus AX70 fluorescence microscope equipped with a TRITC filter for EHD-1 and FITC filter for AH. Whenever possible a minimum of 200 cells per slide

were counted for their acrosomal status. Live and dead cells, whether acrosome reacted or intact, were counted.

2.19. Statistics

All experiments were replicated at least three times and the statistical significance of any differences observed determined by analysis of variance (ANOVA) using the Statview 2 programme (Abacus Concepts Inc., Berkeley, CA). Post hoc testing of differences between group means was accomplished using Fisher's Protected Least Significant Difference (PLSD) with the significance level set at $p < 0.05$.

Chapter Three:

The Impact of Epididymal Maturation on Tyrosine Phosphorylation of Rat Spermatozoa

Chapter 3. The Impact of Epididymal Maturation on Tyrosine phosphorylation of Rat Spermatozoa

3.1. Introduction

As mammalian spermatozoa migrate through the epididymis, they acquire the potential for fertilisation, characterised by acquisition of the ability to express co-ordinated movement and the competence to undergo capacitation. Capacitation is a poorly defined process that is a prerequisite for fertilisation. It was first described in the 1950's (Austin, 1951; Austin, 1952; Chang, 1951; Chang, 1955) and was defined as the time interval of sperm incubation, either *in vivo* or *in vitro* that is required for the final maturation of the spermatozoa to enable them to successfully fertilise an oocyte (Chang, 1984). Subsequently, the ability of spermatozoa to undergo the acrosome reaction in response to contact with the zona pellucida, was included in the definition (Florman and Babcock, 1991; Kopf and Gerton, 1991; Ward and Storey, 1984).

The mechanisms by which epididymal maturation confers upon mammalian spermatozoa the potential to capacitate is poorly understood. In order to address this problem the impact of epididymal maturation on the pattern of tyrosine phosphorylation exhibited by rat spermatozoa has been examined, since this signal transduction pathway is thought to be central to the attainment of a capacitated state (Aitken *et al.*, 1996a; Aitken *et al.*, 1995; Visconti *et al.*, 1995a; Visconti *et al.*, 1995b) and the concomitant expression of hyperactivated motility (Mahoney and Gwathmey, 1999; Si, 1997).

Biological roles of protein kinases are usually mediated by their phosphorylated protein products in intact cells (Greengard, 1978). In order to begin the process of assigning roles to protein kinases present in spermatozoa (Garbers *et al.*, 1973; Hoskins *et al.*, 1972; Hoskins *et al.*, 1974; Lee and Iverson, 1976) it is essential to identify the sperm proteins that are phosphorylated by these enzymes in intact spermatozoa and also to localise these phosphoproteins within the spermatozoon (Chulavatnatol *et al.*, 1982).

While tyrosine phosphorylation during capacitation has been observed in many species (Aitken *et al.*, 1998a; Kalab *et al.*, 1998; Leclerc *et al.*, 1996; Visconti *et al.*, 1995b), the impact of epididymal maturation on this signal transduction pathway has

not yet been ascertained. Specifically it is not known at which point in the cAMP-PKA-tyrosine phosphorylation pathway the epididymis controls the competence of mammalian spermatozoa to undergo capacitation (refer to figure 3.1.). The purpose of this study was to investigate the impact of epididymal maturation on phosphotyrosine expression and its localisation within spermatozoa, using the laboratory rat as an animal model.

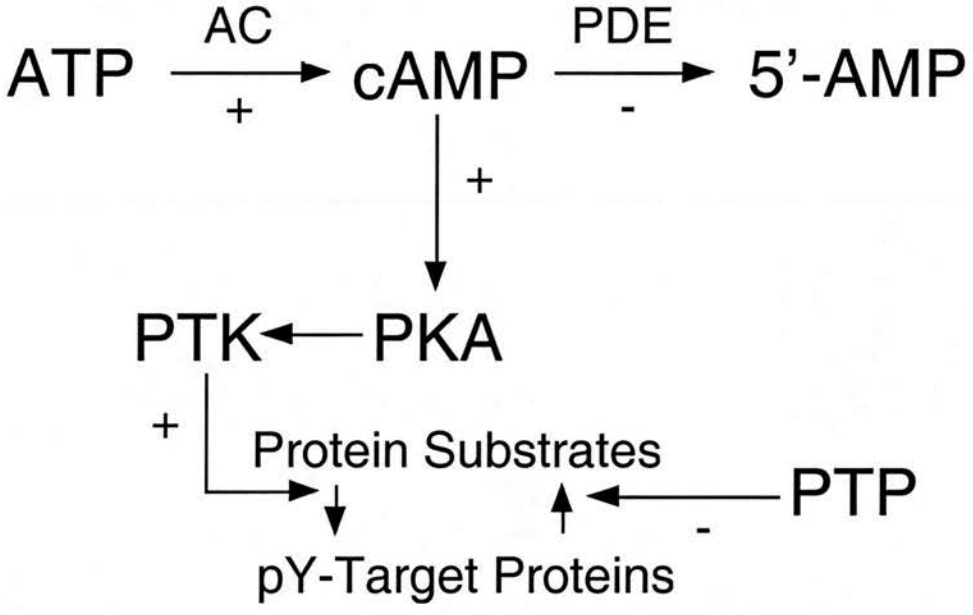


Figure 3.1. This diagram represents a simplified cartoon of the signal transduction pathways involved in the process of tyrosine phosphorylation. ATP is converted into cAMP by the action of adenylyl cyclase (AC). The breakdown of cAMP into 5'-AMP is brought about by the enzymes referred to as phosphodiesterases (PDE). cAMP activates PKA which in turn stimulates protein tyrosine kinases (PTK) to act upon protein substrates resulting in the phosphorylation of tyrosine residues (pY) on target proteins. The dephosphorylation of PKA phosphorylated proteins is induced by protein tyrosine phosphatases (PTP).

3.2. Materials and Methods

Refer to Chapter 2 for general materials and methods.

3.2.i. Time dependent changes in tyrosine phosphorylation in rat epididymal spermatozoa: Western Blot analysis

Rat spermatozoa were extracted from the epididymis as described in Section 2.3., motility and density counts (Sections 2.4. and 2.5. respectively) were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The spermatozoa were incubated in BWW for up to 3 hours, at 37°C.

In order to observe time dependent changes in protein phosphorylation, protein extracts were taken from spermatozoa from the same cell population, at consecutive time intervals. Sperm protein extracts were taken following 0 (i.e. immediately following evaluation of motility and density), 1, 2 and 3 hours incubation. The sperm underwent two different extraction procedures; (i) CHAPS and (ii) SDS in accordance with the methods stated in Section 2.7.

The proteins were separated by SDS-PAGE (Section 2.9.) and analysed by Western Blot analysis (Section 2.11.) to determine the molecular weight of the phosphotyrosine-containing proteins present. Negative controls were set up as described in Section 2.11.i.

3.2.ii. Time dependent changes in tyrosine phosphorylation in rat epididymal spermatozoa: Immunocytochemical analysis

Rat spermatozoa were extracted from the epididymis as described in Section 2.3., motility and density counts were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The spermatozoa were incubated at this concentration in BWW for up to 3 hours at 37°C.

In order to observe time dependent changes in protein phosphorylation in relation to their localisation, slides of spermatozoa were prepared from the same cell population, at consecutive time intervals as stated above in Section 3.2.i. Immunocytochemical analysis was carried out on the slides as described in Section 2.14. as a way of determining where phosphorylated proteins were localised to and also if any changes in localisation occurred over the three hour time period. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14.

Changes were evaluated by counting at least 200 cells per slide for positive staining in specific regions. To determine the proportion of the cell population exhibiting positive staining in different spermatozoal compartments, percentages were calculated from the cell counts.

3.2.iii. Immunolocalisation of tyrosine phosphorylated proteins in rat epididymal spermatozoa *in situ*

Fixation of epididymal tissue was performed by Dr. Richard Sharpe as follows. Animals were perfusion fixed with Bouins' fluid via the dorsal aorta. Bouins' fluid contained 500 ml 40% v/v formaldehyde, 100 ml glacial acetic acid and 2 litres saturated picric acid and it was filtered before use (all of the above reagents were obtained from BDH, UK). The rats were anaesthetised with halothane and the abdominal cavity exposed. A catheter was inserted into the dorsal aorta and the rat perfused initially with 0.9% saline containing 0.01% heparin until the testicular blood vessels cleared followed by Bouins' for 40 minutes, as described previously (Millar *et al.*, 1993). Epididymides were removed, and the tissue was cut into 2.3 mm transverse slices, immersion fixed in Bouins' fluid for 5 hours and transferred to 70% ethanol for storage, before processing for immunohistochemistry.

Tissue was processed through a graded series of alcohols in an automated Shandon processor using a standard 17.5 hour cycle and embedded in paraffin wax. Tissue processing was performed by Mr. Mike Millar.

Glass microscope slides were washed, dried and baked at 300°C for 8 hours. To enhance the adherence of tissue sections, slides were TESPA coated as described in Section 2.17.

Paraffin wax embedded tissue was sectioned to a thickness of 1 μm using a hand operated microtome (Jung RM2035; Leica) and a D-profile knife. Sections were floated onto distilled water, transferred onto treated slides and dried overnight at 50°C prior to use.

When required for use, the sections were dewaxed in Histoclear for five minutes and then rehydrated in the following solvents; (i) absolute ethanol for 20 seconds, (ii) 95% industrial methylated spirits (IMS) for 20 seconds followed by (iii) 70% IMS for 20 seconds. The slides were then washed twice in distilled water for two minutes per wash and fixed with paraformaldehyde as described in Section 2.14.

Immunolocalisation of tyrosine phosphorylated proteins was carried out following fixing as described in Section 2.14. Negative controls were set up whereby the PY20 antibody was replaced with normal mouse serum (refer to Section 2.14.).

3.2.iv. Immunolocalisation of tyrosine phosphorylated proteins in rat epididymal spermatozoa smears

Following dissection from the rat carcass, the epididymal organ was divided into the caput and caudal regions as previously described in Section 2.3. The caput region was gently punctured in several places with a fine needle and protruding sperm were smeared on to several spots of a Hendley slide. This process was repeated on the caudal region using separate slides and immunocytochemical analysis was performed as described in Section 2.14. Negative controls were set up whereby the PY20 antibody was replaced with normal mouse serum (refer to Section 2.14.).

3.2.v. The effect of tyrosine kinase inhibitor genistein on tyrosine phosphorylation in rat epididymal spermatozoa

The epididymides were separated into the caput and caudal regions as normal and each caput from the same animal was sectioned into three separate longitudinal parts. One piece of caput from both epididymides was placed into each of three petri dishes all containing 3 ml BWW. Consequently each petri dish should have contained two pieces of caput epididymis, one from each epididymis from the same animal. Of the three petri dishes, one contained BWW, the second contained BWW that had been supplemented with 10 μM genistein, a known tyrosine kinase inhibitor (Carrera *et al.*, 1996) and the third, contained BWW that had been supplemented with both 10 μM genistein and 100 μM zinc chloride, a tyrosine phosphatase inhibitor.

This was repeated with the caudal sections, motility and density counts were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. Cells were incubated for up to 3 hours incubation at 37°C.

Slides for immunolocalisation of phosphorylated proteins were prepared following incubations of 0 and 3 hours, in accordance with the guidelines set out in Section 2.14.

3.2.vi Measurement of intracellular cAMP levels in rat epididymal spermatozoa

Following the release of rat spermatozoa into BWW as described in Section 2.3., motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml BWW. Spermatozoa were incubated at this concentration in BWW for 3 hours at 37°C. Following completion of the incubation period, extraction of cAMP from the spermatozoa was performed as described in Section 2.15.

3.2.vii Identification of PKA and its subunits in rat epididymal spermatozoa

Rat spermatozoa obtained from the caput and cauda epididymis were incubated at a concentration of 10 x 10^6/ml BWW for 3 hours at 37°C. Proteins were extracted from the cells using 1% SDS as described in Section 2.7. Triplicate lanes of protein from each epididymal region per animal were loaded onto an SDS gel as described in Section 2.9. Following separation of proteins by SDS-PAGE (Section 2.9.) and the transfer of proteins to nitrocellulose by Wet Blot Transfer (Section 2.10.), the membrane was divided into three and probed with mouse monoclonal antibodies against subunits of PKA. There are four distinct regulatory subunits of PKA, the enzyme that catalyses the transfer of the terminal phosphate group from ATP to specific serines or threonines of specific proteins (Alberts *et al.*, 1989): RIα, RIβ, RIIα and RIIβ that define the type I and type II classes of cAMP-dependent protein kinases. The three antibodies used were as follows: anti-PKA (RI subunit used as an immunogen) (250 μg/ml), anti-PKA RIα (250 μg/ml) and anti- PKA RIIα (250 μg/ml) monoclonal antibodies (Affiniti, UK).

Western Blot protocol was carried out as described in Section 2.11. except that PY20 was replaced with one of the PKA antibodies. Each membrane was incubated with 20 ml of the PKA antibody at a 1 in 1000 dilution, which equalled a final concentration of 5 μg/membrane. All three antibodies were probed against sperm proteins extracted from the same animal.

3.3 Results

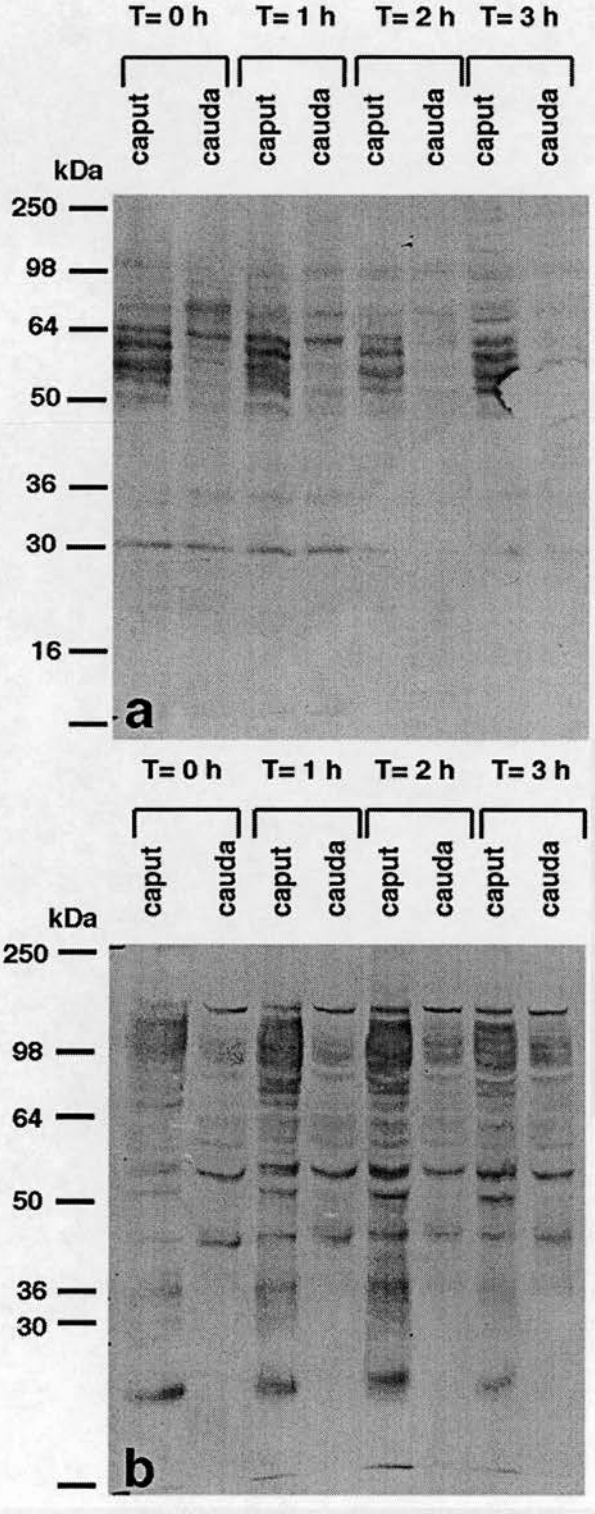
3.3.i. Time dependent changes in tyrosine phosphorylation in rat epididymal spermatozoa: Western Blot analysis

In view of the importance of tyrosine kinase activity in sperm function (Leyton *et al.*, 1992; Leyton and Saling, 1989a; Visconti *et al.*, 1995a; Visconti *et al.*, 1995b), it was of interest to determine the patterns of tyrosine phosphorylation of epididymal rat spermatozoa cultured for variable time intervals in simple defined culture media capable of supporting the capacitation of functionally mature cells.

Rat spermatozoa were incubated in BWW at 37°C for up to 3 hours. Protein was extracted from the spermatozoa at the beginning of the incubation and at 1, 2 and 3 hours thereafter. Western Blot analyses of sperm surface proteins extracted with 1% CHAPS revealed high levels of phosphotyrosine expression in caput epididymal spermatozoa particularly in a cohort of proteins of approximately 50 to 64 kDa (Fig. 3.2a.). The level of phosphorylation of these proteins did not alter significantly over the 3 hour incubation period. In contrast, Western Blot analysis of proteins extracted from caudal spermatozoa under the same conditions revealed low levels of tyrosine phosphorylation in proteins of approximately 60 and 73 kDa (Fig. 3.2a.). Tyrosine phosphorylation of these caudal sperm proteins also decreased over the 3 hour period with maximum intensity observed immediately following extraction from the epididymis.

Following the more severe extraction conditions imposed by SDS (1%), the presence of additional heavily phosphorylated proteins was revealed in caput cells, with molecular weights ranging between 91 and 127 kDa (Fig. 3.2b.). The overall level of tyrosine phosphorylation in caudal spermatozoa was also markedly reduced in these SDS extracted cells in comparison with the caput population with phosphorylation being restricted to proteins of 41, 52 and 127 kDa (Fig. 3.1b.).

Figure 3.2. **(a)** A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% CHAPS. The spermatozoa had previously been incubated in BWW at 37°C for 0, 1, 2 and 3 hours (n = 3). **(b)** A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in BWW at 37°C for 0, 1, 2 and 3 hours (n = 3).


T= 0 h
T= 1 h
T= 2 h
T= 3 h
caput
cauda
kDa
250
98
64
50
36
30
16
a
b

3.3.ii. Time dependent changes in tyrosine phosphorylation in rat epididymal spermatozoa: Immunocytochemical analysis

Immunolocalisation of tyrosine phosphorylated proteins in caput spermatozoa demonstrated heavy labelling over the entire acrosomal domain of the sperm head (Fig. 3.3a.). Approximately 100% of the caput sperm population were found to exhibit this pattern of phosphorylation and this did not change significantly over time (Fig. 3.4 and 3.6). In contrast, protein phosphorylation of caudal epididymal cells was clearly confined to the posterior margin of the acrosome in between 70 and 80% of the sperm population (Fig. 3.3b., 3.5. and 3.6.) although this staining was occasionally accompanied by labelling at the apex of the sperm head in close proximity to the perforatorium (Fig. 3.5a.). The percentage of caudal cells exhibiting phosphorylation at the posterior acrosomal margin remained relatively constant over time (Fig. 3.6.). Tyrosine phosphorylation of the entire acrosomal domain was observed in a small population (10 - 20%) of caudal cells at the beginning of the incubation period (Fig. 3.6.). However the percentage of cells exhibiting this pattern of staining significantly declined over the incubation period to represent less than 5% of the sperm population after 3 hours incubation. The decline in this subpopulation of acrosome labelled caudal epididymal cells could account for the time-dependent decline in tyrosine phosphorylation levels seen in the Western blot analysis (Fig. 3.2a.).

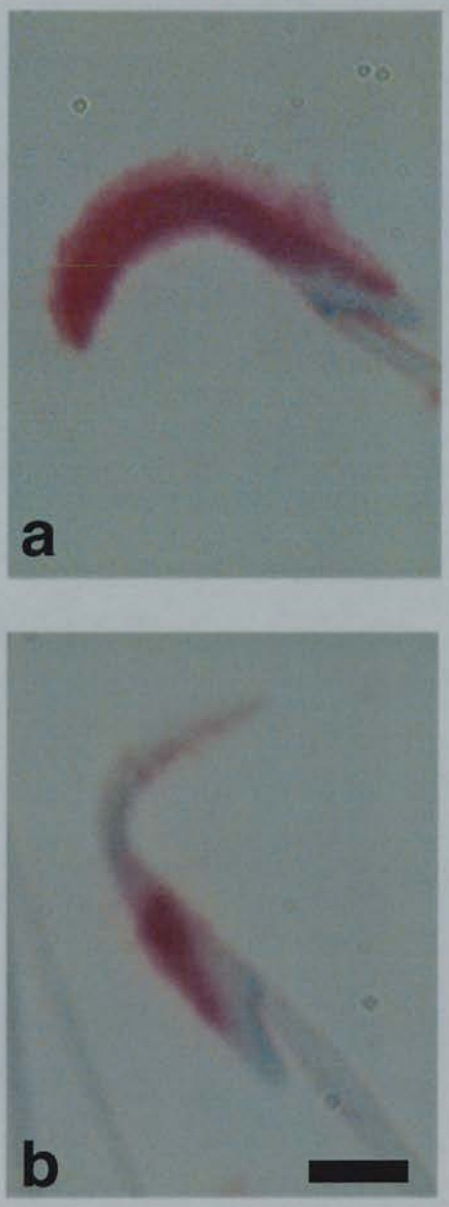


Figure 3.3. Enlarged photomicrographs of **(a)** caput sperm exhibiting tyrosine phosphorylation of the entire acrosomal domain and **(b)** caudal spermatozoa exhibiting tyrosine phosphorylation of the posterior margin of the acrosome. Scale bar = 5 μm.

Figure 3.4. Immunolocalisation of tyrosine phosphorylated proteins in caput epididymal spermatozoa fixed with 1% paraformaldehyde following incubation in BWW at 37°C for 0, 1, 2 and 3 hours (h) (magnification x 1000) (n = 3):

(a) i; caput = 0 h **(a) ii**; caput = 0 h (negative control)

(b) i; caput = 1 h **(b) ii**; caput = 1 h (negative control)

(c) i; caput = 2 h **(c) ii**; caput = 2 h (negative control)

(d) i; caput = 3 h **(d) ii**; caput = 3 h (negative control)

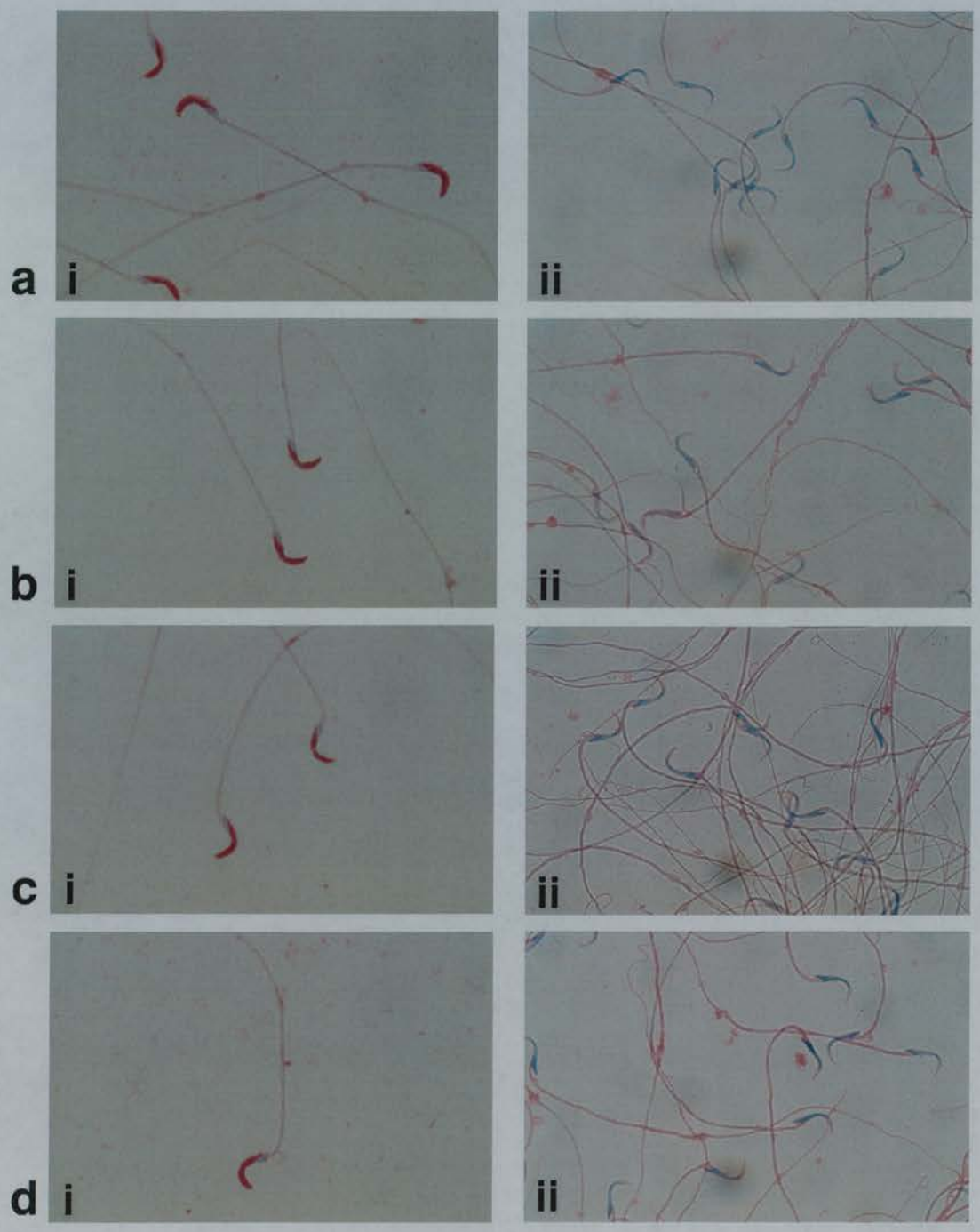

a
i
ii
b
i
ii
c
i
ii
d
i
ii

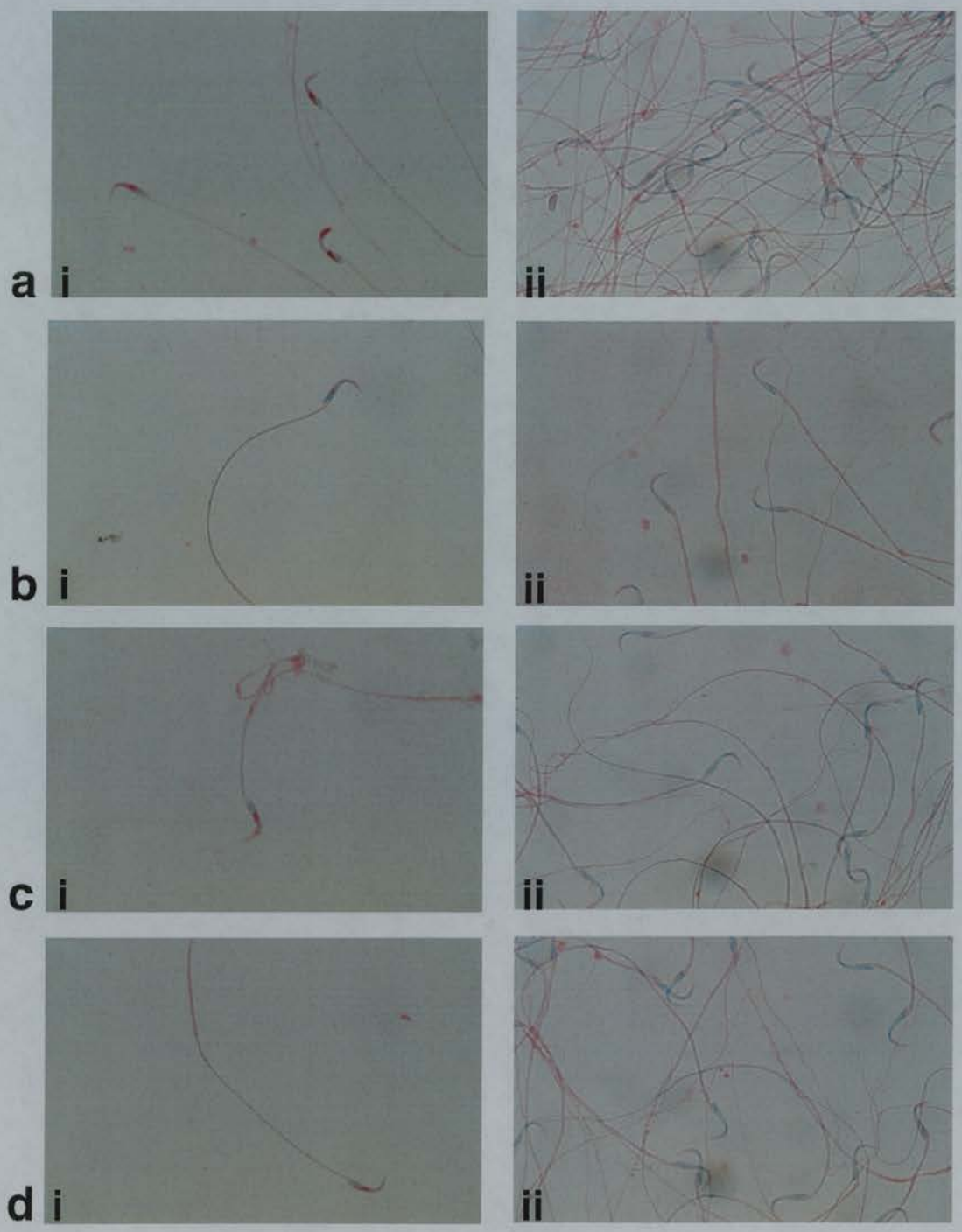
Figure 3.5. Immunolocalisation of tyrosine phosphorylated proteins in caudal epididymal spermatozoa fixed with 1% paraformaldehyde following incubation in BWW at 37°C for 0, 1, 2 and 3 hours (h) (magnification x 1000) (n = 3):

(a) i; cauda = 0 h **(a) ii**; cauda = 0 h (negative control)

(b) i; cauda = 1 h **(b) ii**; cauda = 1 h (negative control)

(c) i; cauda = 2 h **(c) ii**; cauda = 2 h (negative control)

(d) i; cauda = 3 h **(d) ii**; cauda = 3 h (negative control)


a i
ii
b i
ii
c i
ii
d i
ii

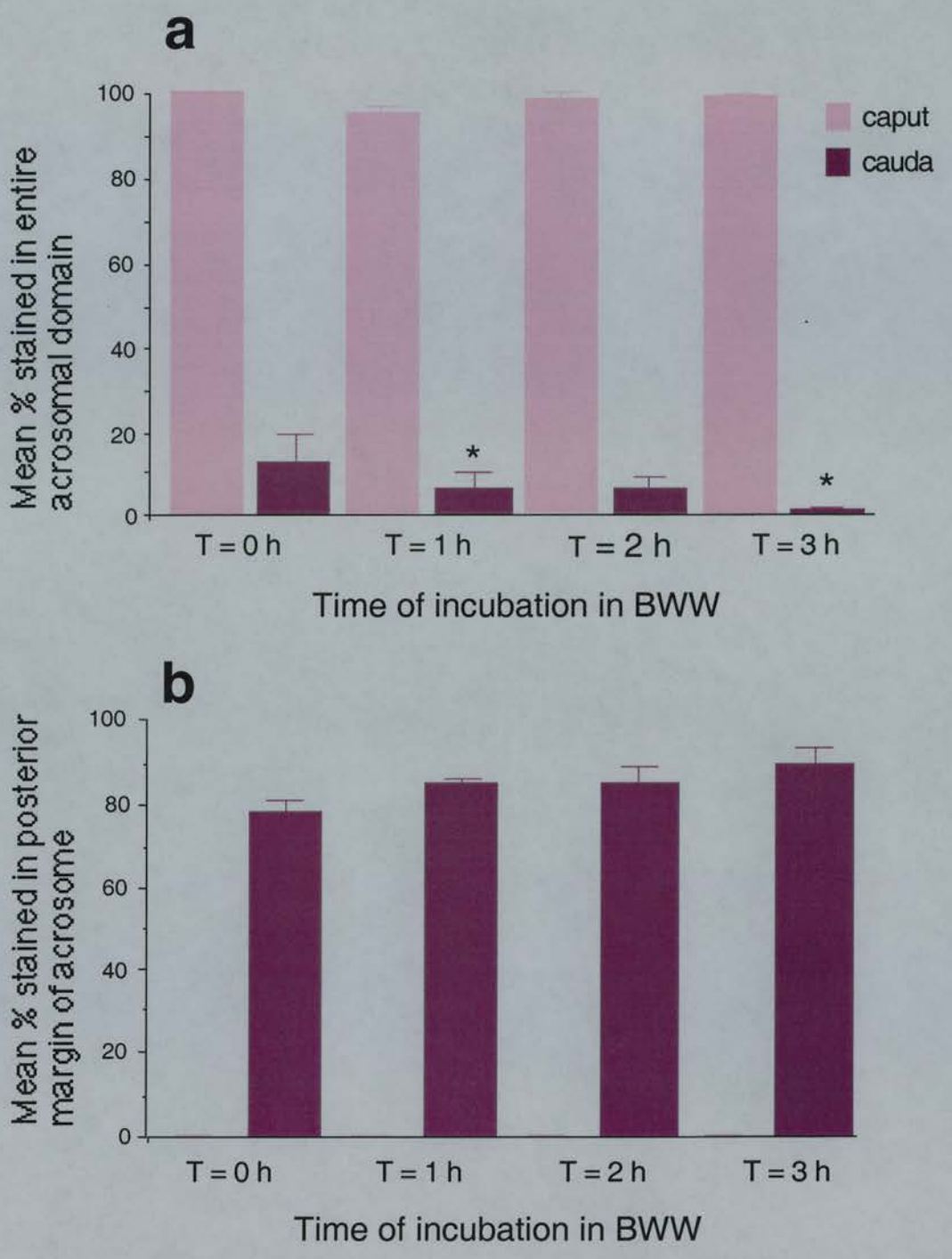


Figure 3.6. Graph representing the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation, following incubation in BWW at 37°C for 0, 1, 2 and 3 hours (n = 3):
(a) entire acrosomal domain **(b)** posterior margin of the acrosome

3.3.iii. Immunolocalisation of tyrosine phosphorylated proteins in rat epididymal spermatozoa *in situ*

With the aim of determining whether or not the phosphorylation patterns observed were reflective of the *in vivo* situation, wax embedded epididymal sections were stained for tyrosine phosphorylation.

Figure 3.7. is representative of caput epididymal sections following immunolocalisation of tyrosine phosphorylated proteins. As demonstrated in figure 3.7a., positive staining of spermatozoa within the tubule lumen of the caput epididymis was observed. On closer examination (Fig. 3.6b.), the precise location of the positive staining was clearly located to the acrosomal domain of head, correlating with the staining observed when caput spermatozoa had been released into BWW prior to imunocytochemical analysis. No positive staining was observed in the negative controls (Fig. 3.7c.).

However, as figure 3.7. clearly demonstrates, not all of the sperm present exhibited positive staining for tyrosine phosphorylation (Fig. 3.7a,b.) and it was also difficult to obtain consistent results using this technique. In addition, no positive staining was detected in any of the caudal epididymal sections.

It was possible that the inability to consistently detect positive staining for phosphorylated proteins was caused by masking of antigens due to the densely packed nature of the sperm cells. Repeats were performed employing modified versions of the above technique. These included microwaving of the slides and incubation in a pressure cooker, in an attempt to unmask the target antigens within the epididymal sections, unfortunately with limited success. Consequently new protocols for the investigation of tyrosine phosphorylation patterns reflective of the *in vivo* situation of rat spermatozoa had to be sought.

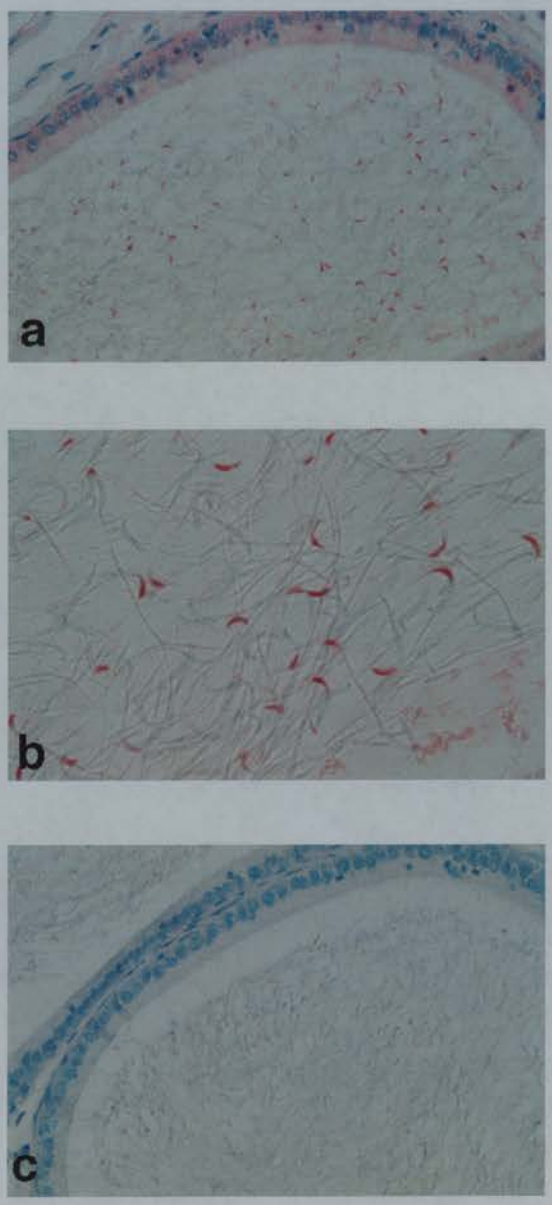


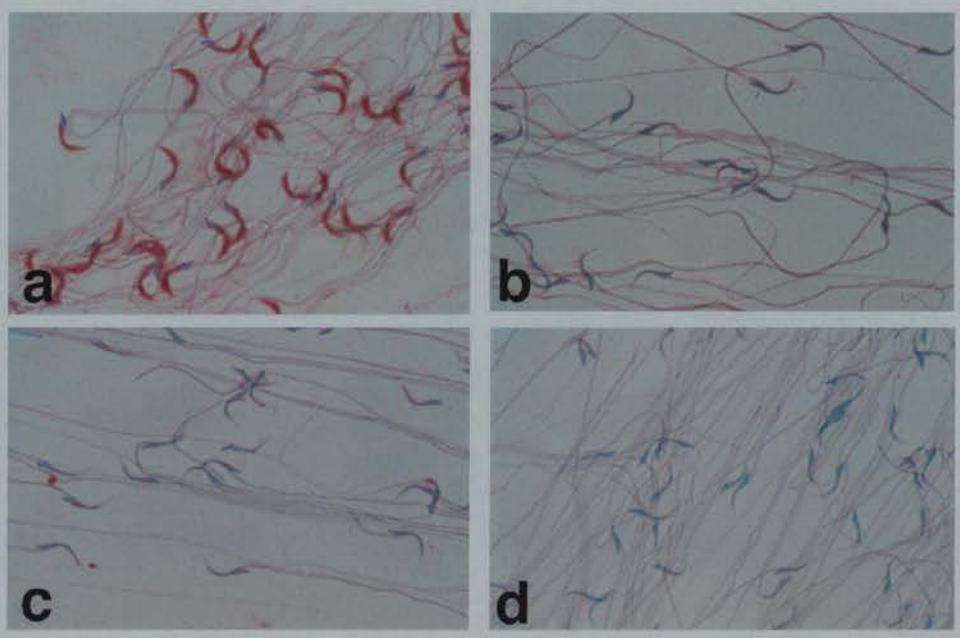
Figure 3.7. Section of caput epididymis stained for tyrosine phosphorylated proteins (n = 1). **(a)** caput epididymis x 200 **(b)** caput epididymis x 1000 **(c)** negative control x 200 (PY20 was replaced with normal mouse serum).

3.3.iv. Immunolocalisation of tyrosine phosphorylated proteins in rat epididymal spermatozoa smears

As an alternative to staining rat epididymal spermatozoa *in vivo* (i.e. wax embedded rat epididymal sections), sperm were extracted from the epididymis as described in Section 3.2.iv. and smeared directly onto a slide, followed by fixing with paraformaldehyde and immunolocalisation of phosphorylated proteins as described in Section 2.14.

Immunocytochemical analysis demonstrated the same patterns of phosphorylation exhibited by rat spermatozoa that had been released into BWW (Fig. 3.8.). Spermatozoa extracted from the caput epididymis clearly demonstrated positive staining for tyrosine phosphorylation over the entire acrosomal domain as shown in Fig. 3.8a. No positive staining could be observed in the negative controls (refer to Fig. 3.8b.). Immunolocalisation also demonstrated that tyrosine phosphorylated proteins in caudal spermatozoa was confined to the posterior margin of the acrosome (Fig. 3.8c.) and no phosphorylation was observed in the negative controls (Fig. 3.8d.).

Figure 3.8. Immunolocalisation of tyrosine phosphorylated proteins in spermatozoa from the epididymis. Spermatozoa were smeared on to slides directly from the caput and caudal regions of the epididymis and then fixed with 1% paraformaldehyde (magnification x 1000) (n = 3). **(a)** Spermatozoa extracted from the caput epididymis. **(b)** Negative control of spermatozoa extracted from the caput epididymis (PY20 was replaced with normal mouse serum). **(c)** Spermatozoa extracted from the cauda epididymis. **(d)** Negative control of spermatozoa extracted from the cauda epididymis (PY20 was replaced with normal mouse serum).


a
b
c
d

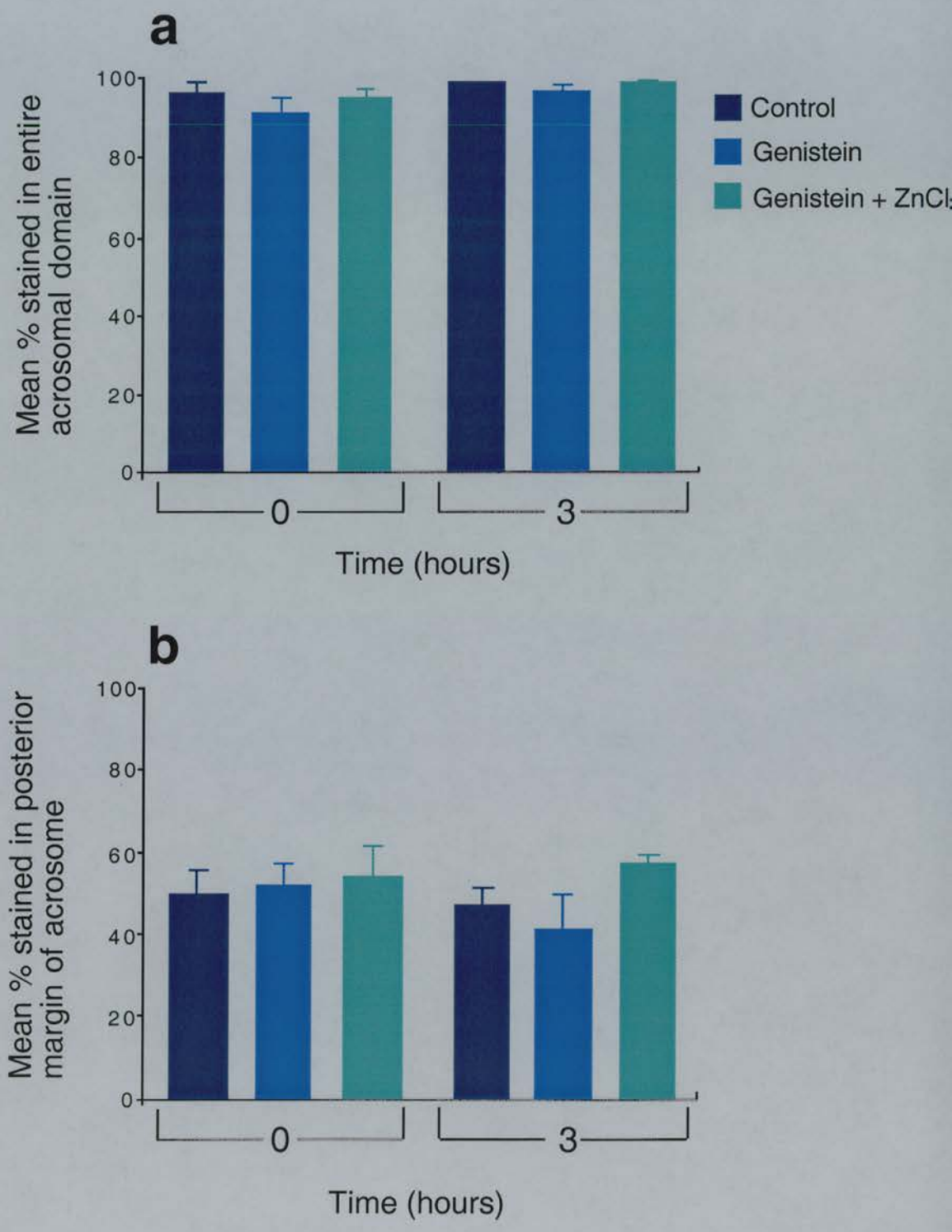
3.3.v. The effect of tyrosine kinase inhibitor genistein on tyrosine phosphorylation in rat epididymal spermatozoa

To determine further whether or not the tyrosine phosphorylation patterns observed were reflective of the *in vivo* situation, or a result of the cells being released and incubated in BWW, spermatozoa were dissected into BWW containing 10 μM genistein, a known tyrosine kinase inhibitor (Carrera *et al.*, 1996). Under these conditions, spontaneous tyrosine phosphorylation as a consequence of sperm release into BWW should be inhibited, whereas any phosphorylation already present should not be affected by the genistein. In addition, sperm were also incubated in BWW supplemented with genistein and zinc chloride, a tyrosine phosphatase inhibitor. The purpose of the inclusion of zinc chloride in addition to genistein to the media was to observe if there was any dephosphorylation of proteins due to the activity of tyrosine phosphatases by comparing these sperm cells with those incubated in normal BWW and BWW supplemented with genistein alone.

The results of this analysis indicated that genistein had no effect on the proportion of cell population exhibiting positive staining in the acrosomal region of caput spermatozoa or the posterior acrosomal margin of caudal spermatozoa (Fig 3.9). This further indicates that the distinct patterns of phosphorylation observed in caput and caudal epididymal spermatozoa *in vitro* were already extant *in vivo*.

In addition, in both caput and caudal spermatozoa, alterations in phosphorylation patterns over the three hour incubation period were limited and dephosphorylation of proteins due to the direct action of protein tyrosine phosphatases was not evident as exemplified by the inability of tyrosine phosphatase inhibitor, zinc chloride to raise levels of tyrosine phosphorylation. In caudal spermatozoa, the presence of zinc chloride did appear to increase the proportion of spermatozoa exhibiting phosphorylation of the posterior acrosomal domain although this was not statistically significant.

Figure 3.9. Graph representing the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation, following incubation in either normal BWW, BWW supplemented with 10 μM genistein or BWW supplemented with both 10 μM genistein and 100 μM zinc chloride (n = 3).
(a) Percentage of caput spermatozoa exhibiting tyrosine phosphorylated proteins in the acrosomal domain. **(b)** Percentage of caudal spermatozoa exhibiting tyrosine phosphorylated proteins in the posterior margin of the acrosome.


a
Mean % stained in entire acrosomal domain
100
80
60
40
20
0
Control
Genistein
Genistein + ZnCl
0
3
Time (hours)
b
Mean % stained in posterior margin of acrosome
100
80
60
40
20
0
0
3
Time (hours)

3.2.vi Measurement of intracellular cAMP levels in rat epididymal spermatozoa

It was of interest to determine whether or not the variation in tyrosine phosphorylation levels between spermatozoa originating from the two different regions of the epididymis was due to variations in intracellular cAMP levels.

There was no statistically significant difference in intracellular cAMP concentration between caput and caudal spermatozoa, although levels tended to be higher in caput cells (Fig. 3.10.). These results indicate that the distinct phosphorylation patterns observed in caput and caudal epididymal spermatozoa *in vivo* and *in vitro* are unlikely to be due to differences in intracellular cAMP alone.

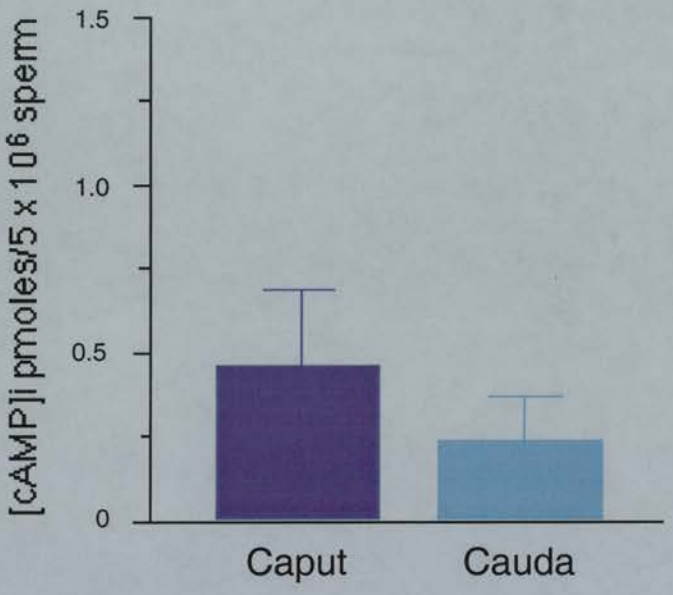


Figure 3.10. Data representing the mean intracellular concentration of cAMP in caput and caudal spermatozoa incubated for 3 hours in BWW at 37°C (n = 6).

3.2.vii Identification of PKA and its subunits in rat epididymal spermatozoa

It is evident that caput and caudal spermatozoa are capable of generating equal levels of cAMP, therefore it was important to determine whether or not the factor inhibiting cAMP action in immature caput epididymal sperm cells could be downstream of cAMP generation. PKA catalyses the transfer of the terminal phosphate group from ATP to specific serines or threonines of selected proteins (Alberts *et al.*, 1989). In the inactive state PKA consists of a complex of two regulatory subunits that bind cAMP and two catalytic subunits. The binding of cAMP alters the conformation of the regulatory subunits, causing them to dissociate from the catalytic units which are consequently activated to phosphorylate specific substrate protein molecules (Alberts *et al.*, 1989). There are four distinct regulatory subunits, RIα, RIβ, RIIα and RIIβ that define the type I and type II classes of cAMP-dependent protein kinases.

Proteins were extracted from rat spermatozoa with 1% SDS following incubation in various treatments for 3 hours at 37°C. Following SDS-PAGE, Western Blot analysis was carried out using three separate PKA antibodies from Affiniti, UK; anti-PKA (RI subunit used as an immunogen), anti-PKA RIα and anti-PKA RIIα. When sperm proteins were probed with the anti-PKA antibody, it was evident that there were equal amounts of PKA in both caput and caudal spermatozoa (Fig. 3.11). There was also no difference in the intracellular presence of the PKA RIα and PKA RIIα subunits in caput and caudal epididymal spermatozoa (Fig. 3.11a,b). However, in both caput and caudal spermatozoa there appeared to be a greater amount of type II PKA than type I (Fig. 3.11a,b).

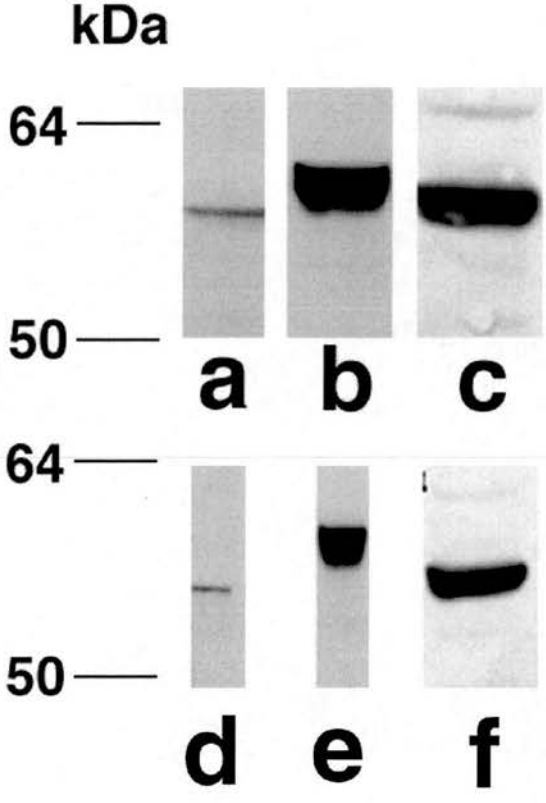


Figure 3.11. Western Blots representing the presence of PKA in caput **(a-c)** and caudal **(d-f)** rat spermatozoa. Spermatozoa were incubated for 3 hours in BWW at 37°C and their proteins extracted with 1% SDS. Following SDS-PAGE the proteins were probed with antibodies against the following subunits of PKA: **(a)** and **(d)** Anti-PKA RIα **(b)** and **(e)** Anti- PKA RIIα **(c)** and **(f)** Anti-PKA (RI subunit used as an immunogen) (n = 3).

3.4. Discussion

The results presented in this chapter highlight how differences in protein tyrosine phosphorylation of rat spermatozoa reflect their maturation status. Phosphotyrosine expression by rat spermatozoa was markedly decreased as they matured in the epididymis, in complete contrast to the situation in the mouse (Visconti *et al.*, 1995a). Thus in the rat, spermatozoa recovered from the caput region of the epididymis exhibited extensive phosphorylation of several proteins exhibiting relative molecular masses of 50-64 kDa and 91-127 kDa following extraction with CHAPS and SDS respectively. Immunocytochemical analysis clearly revealed that these phosphorylated proteins were located in the acrosomal domain of the sperm head.

In complete contrast, Visconti *et al.*, 1995a, found that mouse caput epididymal spermatozoa exhibited low levels of tyrosine phosphorylation, the only phosphorylated band being the p95/116 hexokinase. These authors also demonstrated that subsequent incubation of mouse caput epididymal spermatozoa for prolonged periods of time *in vitro* did not influence the tyrosine phosphorylation status of these cells. Similarly when rat caput epididymal spermatozoa were incubated for up to 3 hours *in vitro*, no change in phosphotyrosine expression was observed.

Tyrosine phosphorylation in rat spermatozoa recovered from the cauda epididymis was greatly reduced relative to caput cells, regardless of the extraction conditions used to recover the proteins. Similarly, Visconti *et al.*, 1995a, found that initial levels of tyrosine phosphorylation in caudal mouse spermatozoa were relatively low and confined to the p95/116 hexokinase, which Kalab *et al.*, 1994 demonstrated to be the major phosphotyrosine-containing protein in mouse sperm membranes. However, when caudal rat and mouse spermatozoa were subsequently incubated in vitro quite different patterns of tyrosine phosphorylation were observed.

In the mouse, tyrosine phosphorylation of a subset of proteins in the range of 40-120 kDa increased gradually over time with maximum levels achieved after 90 min incubation (Visconti *et al.*, 1995a). However in the rat, the overall levels of phosphotyrosine expression became reduced during a 3 hour incubation period *in vitro*. These differences may reflect the contrast between these species in the ability of the spermatozoa to capacitate *in vitro*. Whereas capacitation of rat spermatozoa *in vitro* is extremely difficult, mouse spermatozoa readily engage this process, attaining a full state of capacitation within 90 min as assessed by the ability of the spermatozoa to undergo a ZP-induced acrosome reaction (Florman and Babcock, 1991; Ward and Storey, 1984; Yanagimachi, 1994) as well as their ability to fertilise ZP-intact eggs (Wolf and Inoue, 1976; Yanagimachi, 1994).

Immunocytochemical analyses revealed striking differences between caput and caudal epididymal spermatozoa in the localisation of the phosphotyrosine residues. Whereas extensive staining of the acrosomal region, was observed in virtually all caput spermatozoa, the majority of caudal cells exhibited a narrow band of tyrosine phosphorylation at the posterior acrosomal margin of the sperm head. Evidently, epididymal maturation in the rat is characterised by a progressive loss of phosphotyrosine residues from proteins associated with the acrosome. The caudal cell population also contained a small percentage of spermatozoa exhibiting an extensive caput-like staining of the acrosome, but the cells exhibiting this phosphorylation pattern reduced from 10-20% to <5% over a 3 hour incubation period.

These results suggest that the caudal sperm population is heterogeneous and includes some immature cells that are competent to complete the maturation process when incubated in a simple defined media *in vitro*.

These differences in phosphotyrosine expression between caput and caudal epididymal spermatozoa appeared to reflect the normal *in vivo* situation and were not a consequence of sperm dilution in BWW and subsequent incubation *in vitro*. Thus when the tyrosine kinase inhibitor genistein was incorporated into the media to suppress *de novo* phosphorylations, the characteristic differences in tyrosine phosphorylation between caput and caudal epididymal spermatozoa were still observed. The fact that the addition of zinc chloride to the BWW media supplemented with genistein, did not significantly increase the proportion of sperm cells exhibiting phosphorylation in comparison with most of the other treatments, suggested that the activity of protein tyrosine phosphatases under these conditions was relatively low. If tyrosine phosphatase activity was high then a reduction in phosphorylation could have been expected in those spermatozoa incubated in BWW supplemented with genistein alone. As genistein prevented further *de novo* phosphorylations in BWW, then it is possible to assume that highly active protein tyrosine phosphatases would have lead to the dephosphorylation of tyrosine residues phosphorylated *in vivo*.

Confirmation that the differences in tyrosine phosphorylation between caput and caudal epididymal spermatozoa exist *in vivo*, came from immunocytochemical analyses of spermatozoa sampled directly from the epididymis without being diluted in medium BWW.

The purpose of these changes in tyrosine phosphorylation are unknown but could be related to the control of acrosomal exocytosis. Caput epididymal spermatozoa are incapable of undergoing the acrosome reaction even when they are exposed to high concentrations of the divalent cation ionophore, A23187. As spermatozoa mature they acquire a capacity to undergo the acrosome reaction in response to a calcium stimulus

in concert with the progressive dephosphorylation of proteins in the sperm head. It is possible that these phosphoproteins negatively regulate sperm function by suppressing the membrane fusion events associated with the acrosome reaction. In order to address this hypothesis ultrastructural data is clearly required to determine the precise localisation of the phosphotyrosine residues on the sperm head in relation to the membrane fusion events associated with the acrosome reaction.

In terms of the cAMP/PKA kinase pathway, there appeared to be no significant differences between caput and caudal epididymal spermatozoa in the availability of intracellular cAMP. In addition Western blot analysis indicated that there were no differences between caput and caudal epididymal spermatozoa in the presence of PKA subunits of RI, RIα and RIIα, both caput and caudal spermatozoa possessing a greater amount of RIIα than RIα. This is in accordance with previous studies indicating that in rat and bovine spermatozoa the regulatory subunit (RII) of a type II cAMP-dependent protein kinase is tightly associated with the sperm flagellum (Horowitz *et al.*, 1984; Horowitz *et al.*, 1989; Horowitz *et al.*, 1988). Lieberman (Lieberman *et al.*, 1988) also demonstrated that a type II cAMP-dependent protein kinase was located at the outer mitochondrial membrane of bovine sperm. They suggested that it may play a role in the direct cAMP-mediated stimulation of mitochondrial respiration during sperm activation. Pariset and Weinman (Pariset and Weinman, 1994), found two isoforms of the regulatory subunit RIIα of cAMP-dependent protein kinase in human sperm, one of which was localised to the microtubules and the other was detected among the cytoplasmic proteins. Additionally Macleod (MacLeod *et al.*, 1994) localised RII and its binding proteins to the fibrous sheath of rat sperm flagellum.

If the cAMP/PKA pathway is functional at all stages of epididymal maturation it suggests that the changes observed in this study are likely to be due to differences in phosphatase activity directed against the PKA activated tyrosine kinase.

In conclusion, the results presented in this chapter indicate that epididymal maturation of rat spermatozoa is associated with a progressive loss of tyrosine phosphorylated proteins from the acrosomal domain of the sperm head. These findings provide us with valuable biochemical markers to further elucidate the mechanisms of epididymal maturation. Specifically these studies provide important clues as to the impact of epididymal maturation on tyrosine phosphorylation, a process which is associated with the competence for both movement and capacitation of mammalian spermatozoa, as they transit the epididymis and become programmed for fertilisation.

Chapter Four:

The Role of cAMP in Tyrosine Phosphorylation of Rat Epididymal Spermatozoa

Chapter 4. The Role of cAMP in Tyrosine Phosphorylation of Rat Epididymal Spermatozoa

4.1. Introduction

The regulation of tyrosine phosphorylation in mature mammalian spermatozoa is thought to be via a cAMP/protein kinase A (PKA) pathway that appears to be unique to this cell type.

cAMP is synthesised from ATP by a plasma-membrane-bound enzyme known as adenylyl cyclase. In turn, cAMP is rapidly and continuously broken down by one or more cAMP phosphodiesterases, which hydrolyse cAMP to adenosine 5'-monophosphate (5'-AMP). cAMP activates PKA, the enzyme that uniquely in spermatozoa is responsible for stimulating protein tyrosine kinase (PTK) activity, which then phosphorylates key tyrosine residues on target proteins (refer overleaf for Fig. 4.1.).

As it is often important for the effects of cAMP to be transient, mechanisms are in place to induce the rapid dephosphorylation of proteins phosphorylated by PKA. Such mechanisms include the activity of protein tyrosine phosphatases (PTP) (Fig. 4.1.).

Thus protein tyrosine phosphorylation in capacitating boar (Kalab *et al.*, 1998), mouse (Visconti *et al.*, 1995b) and human spermatozoa has been found to be cAMP-dependent and inhibited by antagonists of this system (Aitken *et al.*, 1998a; Leclerc *et al.*, 1996; Visconti *et al.*, 1995b). Mahoney and Gwathmey (Mahoney and Gwathmey, 1999), also demonstrated in *Macaca fascicularis* that tyrosine phosphorylation of sperm tail proteins is an integral signalling pathway modulating some, but not all, of the motion characteristics associated with cAMP and caffeine-stimulated hyperactivation. A 55 kDa protein tyrosine phosphorylated in bovine spermatozoa was also found to be regulated by cAMP and associated with motility (Brandt and Hoskins, 1980; Vijayaraghavan *et al.*, 1997).

The purpose of this chapter was to investigate the involvement of a cAMP/PKA pathway in the regulation of tyrosine phosphorylation. In addition the impact of epididymal maturation on the signal transduction pathways regulating this process in rat spermatozoa was determined.

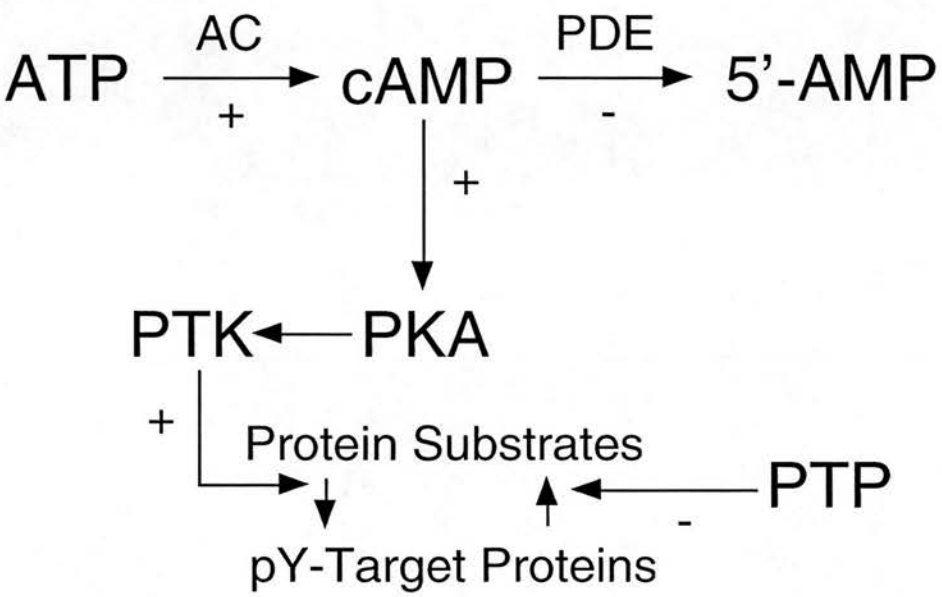


Figure 4.1. This diagram represents a simplified cartoon of the signal transduction pathways involved in the process of tyrosine phosphorylation. ATP is converted into cAMP by the action of adenylyl cyclase (AC). The breakdown of cAMP into 5'-AMP is brought about by the enzymes referred to as phosphodiesterases (PDE). cAMP activates PKA, which in turn stimulates protein tyrosine kinases (PTK) to act upon protein substrates resulting in the phosphorylation of tyrosine residues (pY) on target proteins. The dephosphorylation of PKA phosphorylated proteins is induced by protein tyrosine phosphatases (PTP).

4.2. Materials and Methods

Refer to Chapter 2 for general materials and methods.

4.2.i. The effect of increased intracellular cAMP levels on tyrosine phosphorylation: Western Blot analysis

Rat spermatozoa were extracted from the epididymis as described in Section 2.3., motility and density counts (Sections 2.4. and 2.5. respectively) were performed and the concentration of each cell population adjusted to 10 x 10^6/ml.

Intracellular cAMP levels were elevated in rat spermatozoa by incubating the cells with the following reagents: pentoxifylline (PTX), an inhibitor of phosphodiesterase, the enzyme responsible for the breakdown of cAMP into 5' AMP and N6, 2-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP), a membrane permeant analogue of cAMP. Spermatozoa in BWW were incubated with these two reagents at various concentrations either singularly or in combination, at a temperature of 37°C for 3 hours.

On completion of the incubation period, sperm protein extracts were taken following 3 hours incubation. The sperm underwent two different extraction procedures using the following detergents: (i) CHAPS and (ii) SDS in accordance with the methods stated in Section 2.7.

The proteins were separated by SDS-PAGE (Section 2.9.) and analysed by Western Blot analysis (Section 2.11.) to determine the molecular weight of the phosphotyrosine-containing proteins present.

4.2.ii. The effect of increased intracellular cAMP levels on tyrosine phosphorylation: Immunocytochemical analysis

Rat spermatozoa were extracted from the epididymis as described in Section 2.3., motility and density counts were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The spermatozoa were incubated with PTX and dbcAMP as described in Section 4.2.i. above

Immunocytochemical analysis was carried out on the slides as described in Section 2.14. to determine the localisation of tyrosine phosphorylated proteins. Both paraformaldehyde and methanol fixing of rat spermatozoa were compared as described in Section 2.14. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14. An additional control involved the replacement of

PY20 with a monoclonal antibody against β-catenin (n = 1) as explained in Section 2.14.

Changes were evaluated by counting at least 200 cells per slide for positive staining in specific regions. To determine the proportion of the cell population exhibiting positive staining in different compartments, percentages were calculated from the cell counts.

4.2.iii The effect of PTX on intracellular cAMP levels in rat epididymal spermatozoa

Following the release of rat spermatozoa into BWW as described in Section 2.3., motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. Spermatozoa in BWW were incubated at this concentration with 3 mM PTX for 3 hours at 37°C. Following completion of the incubation period, extraction of cAMP from the spermatozoa was performed as described in Section 2.15.

4.2.iv PKA inhibition: The effect of PKA inhibitor H89 on tyrosine phosphorylation

H89, a potent and specific inhibitor of PKA, was incorporated into BWW at a final concentration of 10 μM. Spermatozoa obtained from one epididymal organ were released into normal BWW and those from the remaining epididymis released into BWW supplemented with 10 μM H89. Motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. Spermatozoa were then incubated with 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C, followed by extraction of the proteins with 1% SDS as described in Section 2.7.

The proteins were separated by SDS-PAGE (Section 2.9.) and analysed by Western Blot analysis (Section 2.11.) to determine the molecular weight of the phosphotyrosine-containing proteins.

4.2.v Tyrosine kinase inhibition: The effect of tyrosine kinase inhibitor genistein on tyrosine phosphorylation

The epididymides were separated into the caput and caudal regions as described in Section 2.3. Each caput section was placed in a separate petri dish, one of which contained normal BWW while the other contained BWW that had been supplemented with 10 μM genistein, a known tyrosine kinase inhibitor (Carrera *et al.*, 1996). The spermatozoa were released into their respective media as stated in Section 2.3.

This was repeated with the caudal sections, motility and density counts were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. Cells were incubated for 3 hours with 3 mM PTX and 5 mM dbcAMP at 37°C.

On completion of the 3 hour incubation period, slides for immunocytochemical analysis were prepared in accordance with the guidelines set out in Section 2.14. In addition, protein extracts were obtained using 1% SDS, followed by SDS-PAGE and Western Blot analysis (refer to Sections 2.9.- 2.11.).

4.3 Results

4.3.i. The effect of increased intracellular cAMP levels on tyrosine phosphorylation: Western Blot analysis

Tyrosine phosphorylation in spermatozoa has been widely studied in many species including human (Aitken *et al.*, 1996a; Aitken *et al.*, 1995; Carrera *et al.*, 1996; Emiliozzi and Fenichel, 1997), murine (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b), bovine (Brandt and Hoskins, 1980; Vijayaraghavan *et al.*, 1997) and the sea urchin (Bookbinder *et al.*, 1991), as it is known to be central to the attainment of the capacitated state (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b). Since it is well established that cAMP plays a pivotal role in the regulation of tyrosine phosphorylation (Bookbinder *et al.*, 1991; Brandt and Hoskins, 1980; Kalab *et al.*, 1998; Leclerc *et al.*, 1996; Mahoney and Gwathmey, 1999; Vijayaraghavan *et al.*, 1997a; Visconti *et al.*, 1995b) it was of interest to investigate this signal transduction pathway during epididymal maturation in the rat.

Intracellular cAMP levels were raised in rat spermatozoa by the addition of the phosphodiesterase inhibitor, PTX and also the membrane permeant analogue of cAMP, dbcAMP. Rat spermatozoa were incubated for 3 hours at 37°C in BWW, with various concentrations of PTX and dbcAMP and then their proteins extracted with 1% SDS. In both caput and caudal epididymal spermatozoa, it was evident that tyrosine phosphorylation was significantly up-regulated following treatment with these two reagents, although these effects were much more marked in caudal cells (Fig. 4.2.). Treatment of caudal spermatozoa with both PTX and dbcAMP induced tyrosine phosphorylation in a new set of proteins with molecular weights of approximately 58, 62, 79, 98, 115, 125, 153 and 205 kDa. Up-regulation of phosphorylation was also observed in proteins of approximately 52, 64 and 84 kDa. Increased phosphorylation was particularly evident with a combination of 3 mM PTX and 5 mM dbcAMP (Fig. 4.2b.). Tyrosine phosphorylation was also induced in caput spermatozoa under the same conditions, although the effect observed was not as significant as found with caudal cells (Fig. 4.2a.). An increase in tyrosine phosphorylation of proteins ranging from approximately 28-33 kDa and 60, 84 and 120 kDa was observed in caput spermatozoa treated with PTX and dbcAMP and phosphorylation of a new protein of approximately 215 kDa was also induced. These effects were not as clearly dose dependent as observed in caudal cells.

A similar picture emerged when instead of using SDS as a solubilisation agent, cytosolic and membrane proteins were extracted with CHAPS. Elevation of intracellular cAMP by the combination of dbcAMP and PTX specifically enhanced the phosphorylation of three high molecular weight proteins of approximately 98, 115 and 125 kDa in caudal cells extracted with CHAPS (Fig. 4.3.). These proteins were also phosphorylated in caput spermatozoa following stimulation of intracellular cAMP levels although not to the same extent as observed in the caudal cells. In addition, elevated intracellular cAMP induced the phosphorylation of a group of proteins in the range of 50 to 64 kDa in caput but not caudal cells (Fig. 4.3.). The fact that PTX had to accompany the dbcAMP in order to induce high levels of tyrosine phosphorylation in both caput and caudal cells suggests the presence of highly active intracellular phosphodiesterases at all stages of epididymal maturation (Fig. 4.3.).

Figure 4.2. **(a)** A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput spermatozoa with 1% SDS. Prior to extraction, the spermatozoa were incubated with various concentrations of PTX and dbcAMP in BWW at 37°C for 3 hours (n = 4).

(b) A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caudal spermatozoa with 1% SDS. Prior to extraction, the spermatozoa were incubated with various concentrations of PTX and dbcAMP in BWW at 37°C for 3 hours (n = 4).

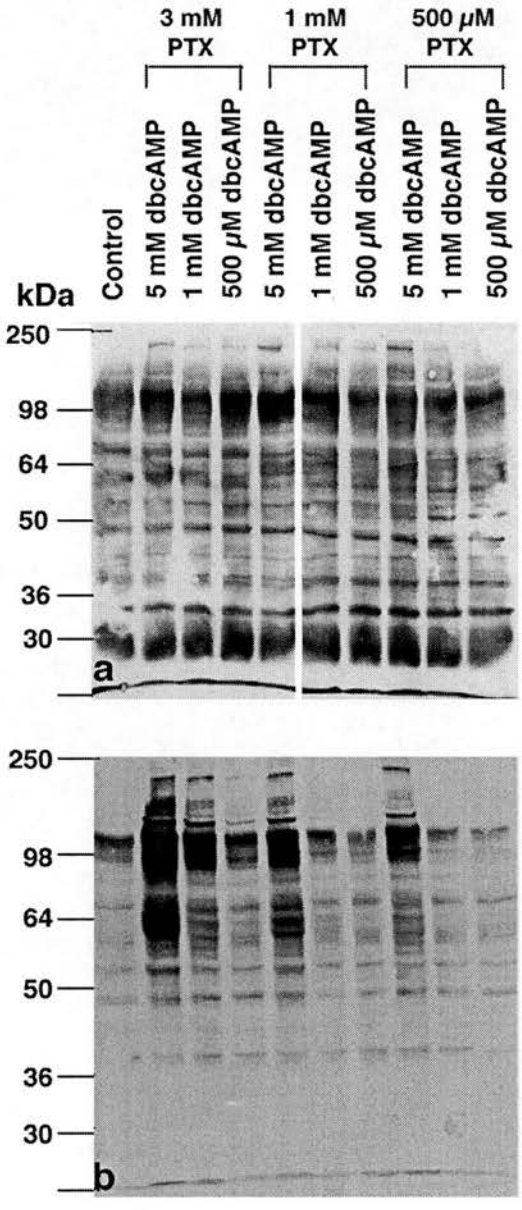
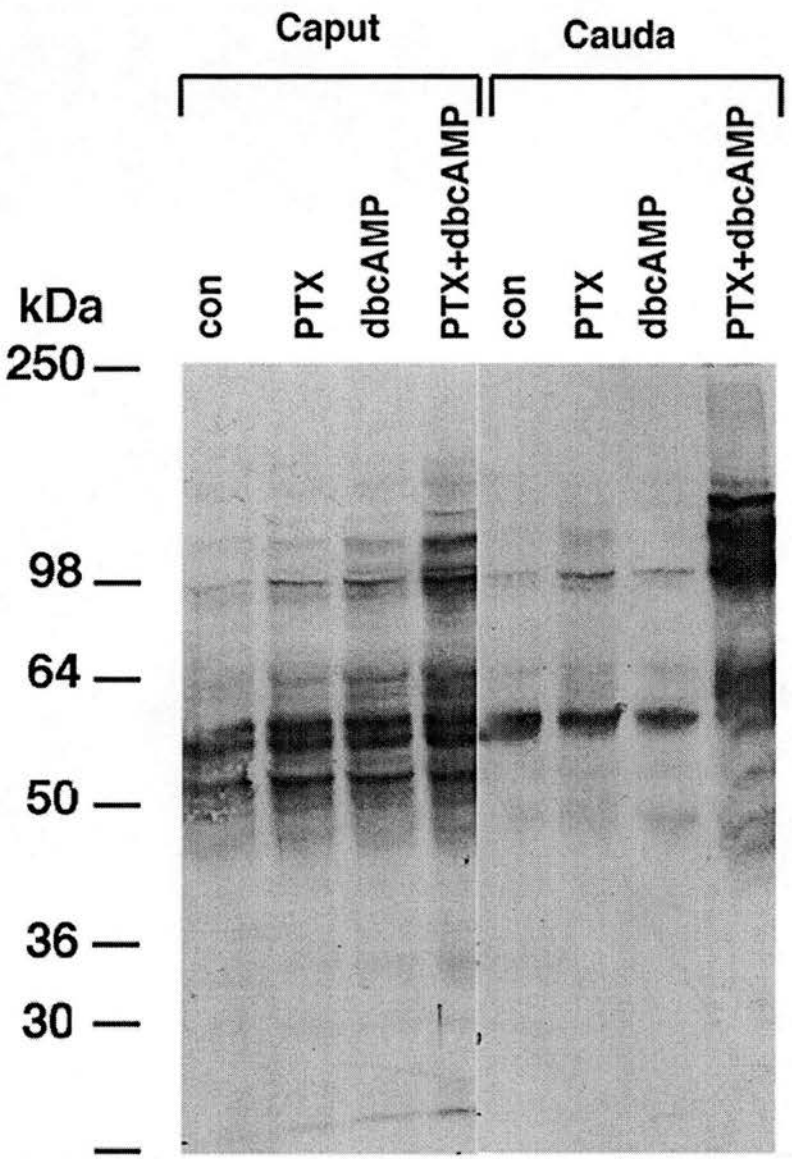

3 mM PTX
1 mM PTX
500 μM PTX
Control
5 mM dbcAMP
1 mM dbcAMP
500 μM dbcAMP
5 mM dbcAMP
1 mM dbcAMP
500 μM dbcAMP
5 mM dbcAMP
1 mM dbcAMP
500 μM dbcAMP
kDa
250
98
64
50
36
30
a
250
98
64
50
36
30
b

Figure 4.3. A Western Blot of proteins probed with an antibody against phosphotyrosine conjugated with HRP, following extraction from caput and caudal spermatozoa with 1% CHAPS. The spermatozoa had been incubated with 5mM dbcAMP and 3mM PTX in BWW at 37°C for 3 hours (n = 3).


Caput
Cauda
kDa
con
PTX
dbcAMP
PTX+dbcAMP
con
PTX
dbcAMP
PTX+dbcAMP
250
98
64
50
36
30

4.3.ii. The effect of increased intracellular cAMP levels on tyrosine phosphorylation: Immunocytochemical analysis

Immunocytochemistry demonstrated that in caudal cells only, stimulation by dbcAMP in the presence of PTX lead to the phosphorylation of a new set of proteins located to the tail in up to 80% of the sperm population (Fig 4.4. - 4.6.). The percentage of each sperm population demonstrating this positive tail stain varied significantly between treatments (Fig. 4.6.). Populations of caudal spermatozoa treated with PTX or dbcAMP alone contained a significantly smaller population of cells demonstrating tyrosine phosphorylated tails, than those treated with dbcAMP combined with PTX, in accordance with the results of the Western Blot analysis (Fig. 4.3 and 4.6a.). However, with immature caput spermatozoa treated under the same conditions, it was evident that tyrosine phosphorylated proteins remained restricted to the acrosomal domain, with less than 3% demonstrating any tail phosphorylation (Fig. 4.6a.).

As expected, when rat caudal spermatozoa were treated with varied doses of PTX and dbcAMP, there was a dose-dependent stimulation of spermatozoa exhibiting positive staining of the tail (Fig. 4.5b.). In contrast, there was no significant difference observed between caput spermatozoa treated with different doses of PTX and dbcAMP (Fig. 4.5a.). No increase in the percentage population of spermatozoa exhibiting phosphorylation of the entire acrosomal domain was evident in either caput or caudal spermatozoa (Fig. 4.5c,d.).

In order to determine whether or not the method of fixing exerted any effect on the immunolocalisation of tyrosine phosphorylated proteins, spermatozoa obtained from the same population of cells were either fixed with methanol or paraformaldehyde as described in Section 2.14. There was no statistically significant difference in the percentage population of spermatozoa exhibiting positive staining of the tail following the two different methods of fixing (Fig. 4.6b.). Microscopic analysis revealed no differences in any of the patterns of staining, whether located to the entire acrosomal domain, posterior acrosomal margin or tail.

Figure 4.4. Immunolocalisation of tyrosine phosphorylated proteins in caput and caudal spermatozoa fixed with paraformaldehyde, following incubation with 3mM PTX and 5mM dbcAMP in BWW at 37°C for 3 hours (n = >12). For the negative controls, PY20 was replaced with an antibody against β-catenin (n = 1) or normal mouse serum (n = >12) (magnification x 1000):
(a) caput control **(b)** caput 3mM PTX + 5mM dbcAMP **(c)** caput control (negative control) **(d)** caput 3mM PTX + 5mM dbcAMP (negative control) **(e)** cauda control **(f)** cauda 3mM PTX + 5mM dbcAMP dbcAMP **(g)** cauda control (negative control) **(h)** cauda 3mM PTX + 5mM dbcAMP (negative control).

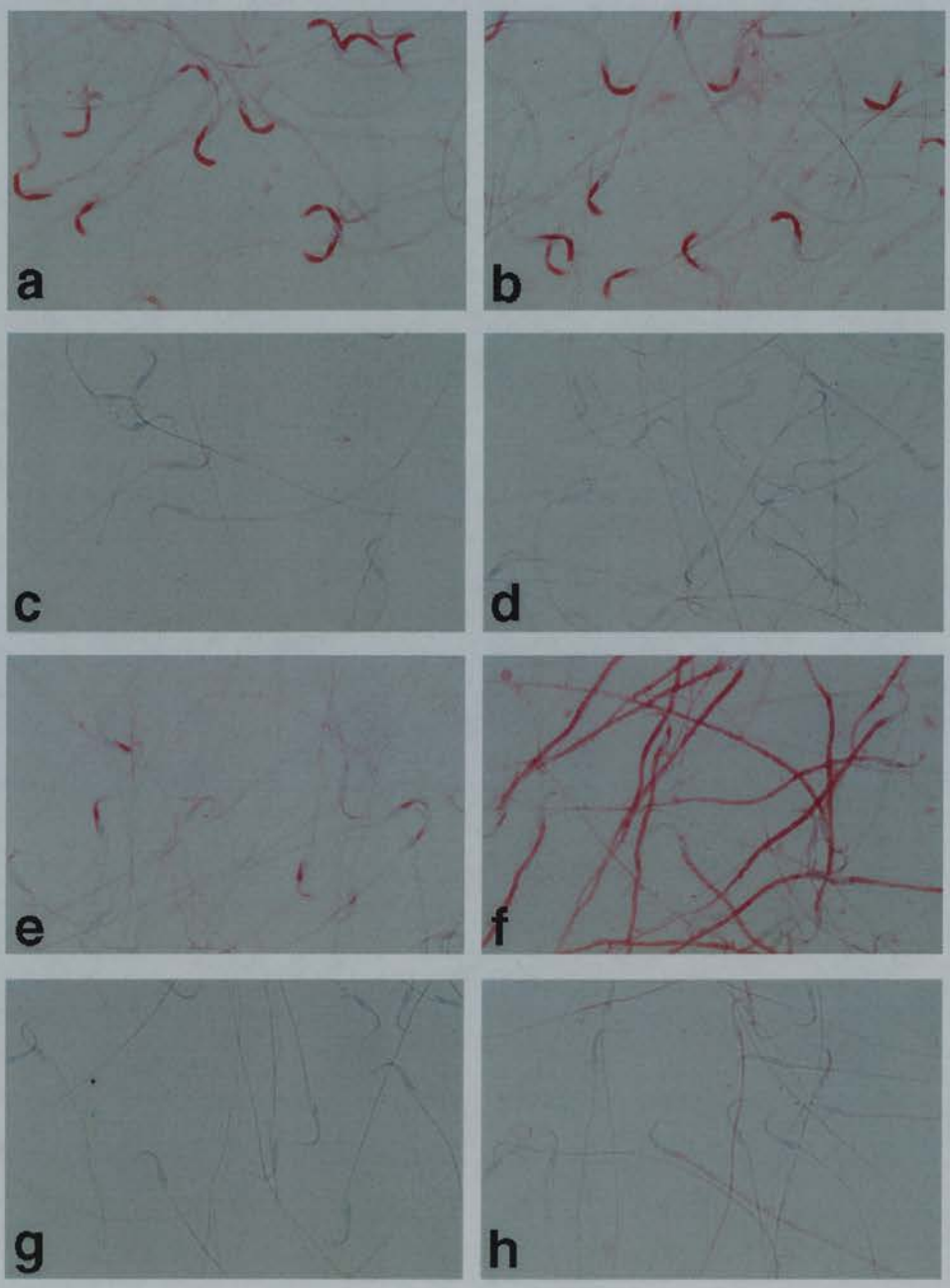
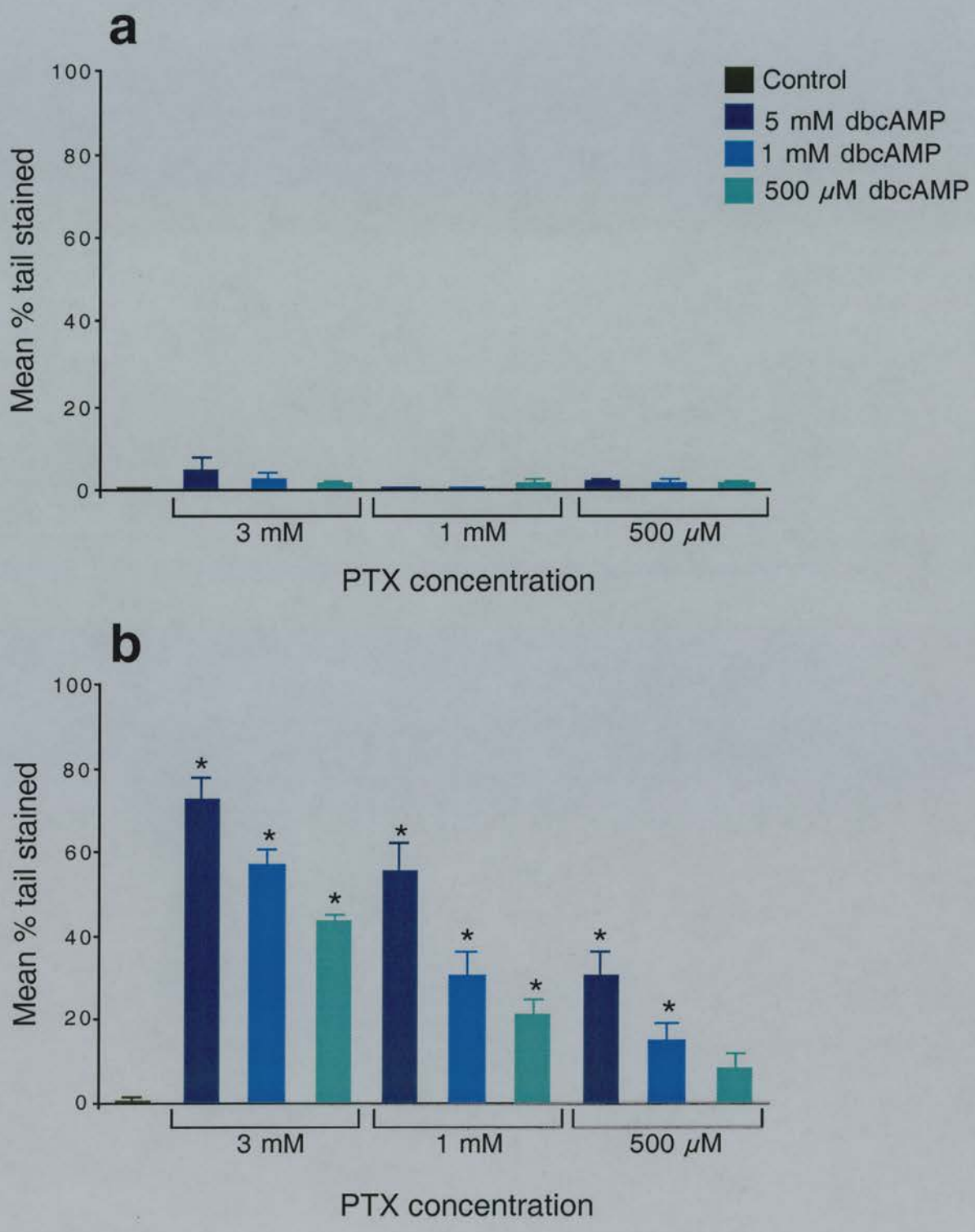
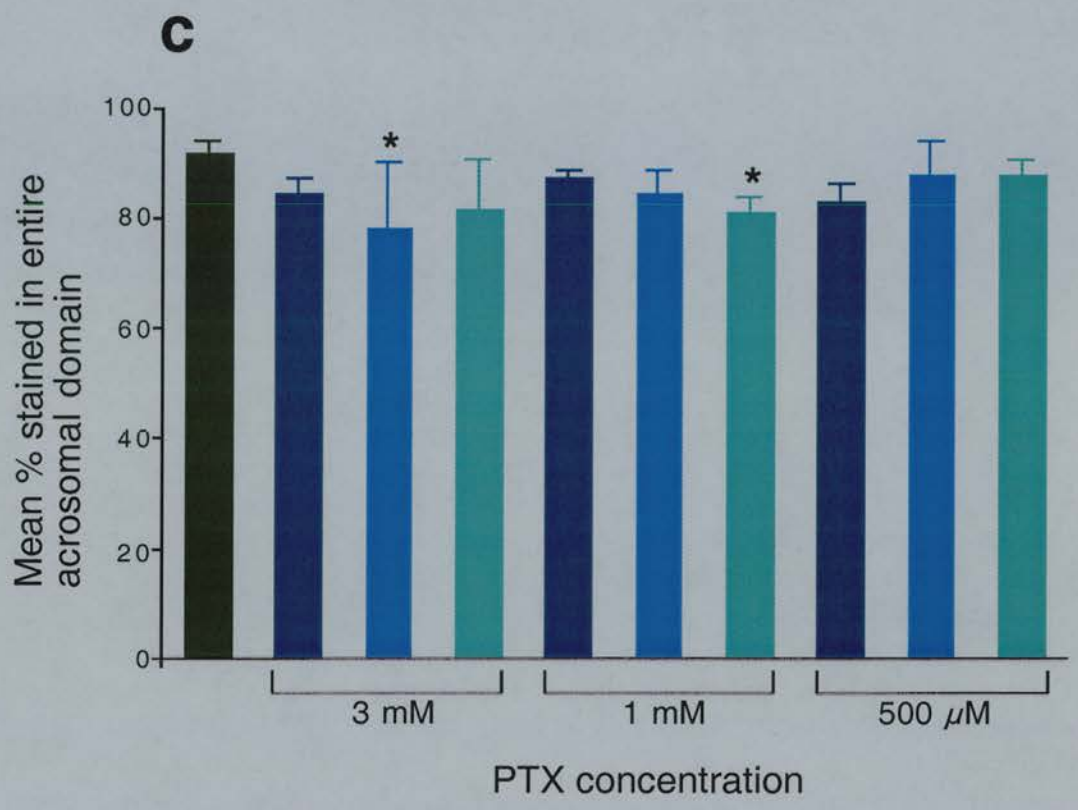

a
b
c
d
e
f
g
h

Figure 4.5. Graphical representation of the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation, following incubation with various concentrations of PTX and dbcAMP in BWW at 37°C for 3 hours (paraformaldehyde fixed) (n = 3):
(a) caput spermatozoa (tail) **(b)** cauda spermatozoa (tail) **(c)** caput spermatozoa (entire acrosomal domain) **(d)** cauda spermatozoa spermatozoa (entire acrosomal domain).


a
Control
5 mM dbcAMP
1 mM dbcAMP
500 μM dbcAMP
Mean % tail stained
100
80
60
40
20
0
3 mM
1 mM
500 μM
PTX concentration
b
Mean % tail stained
100
80
60
40
20
0
*
3 mM
1 mM
500 μM
PTX concentration


c
100
80
60
40
20
0
Mean % stained in entire acrosomal domain
*
*
3 mM
1 mM
500 μM
PTX concentration

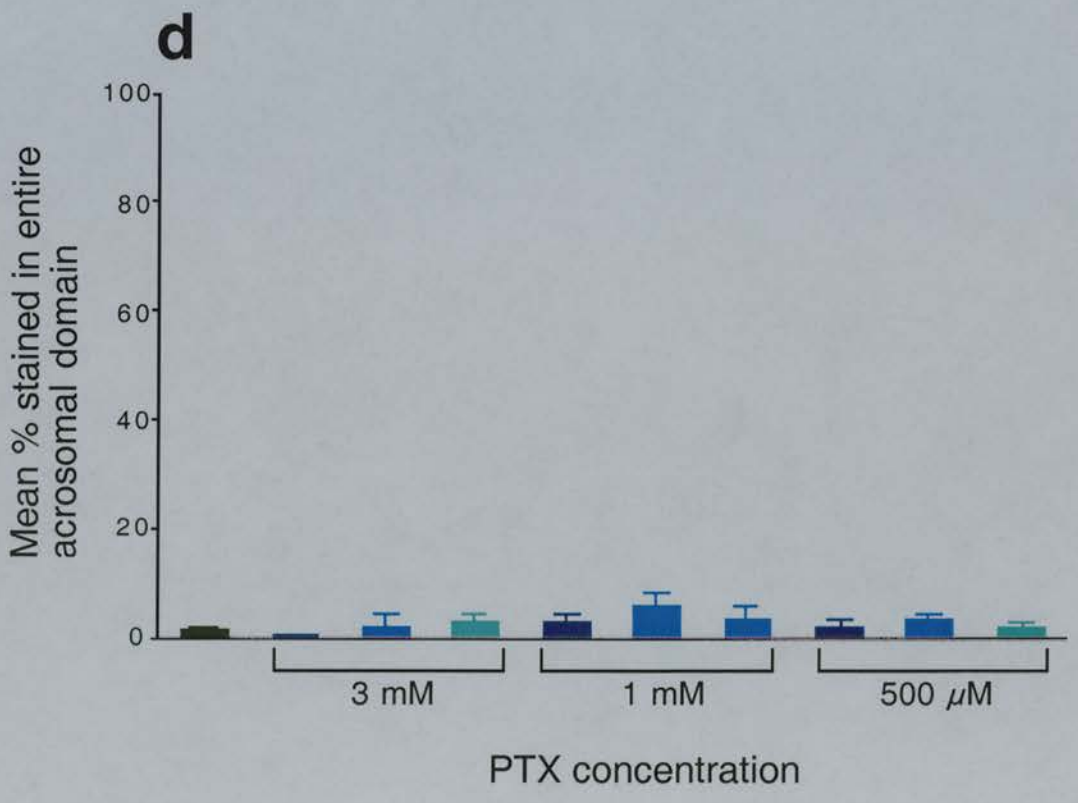
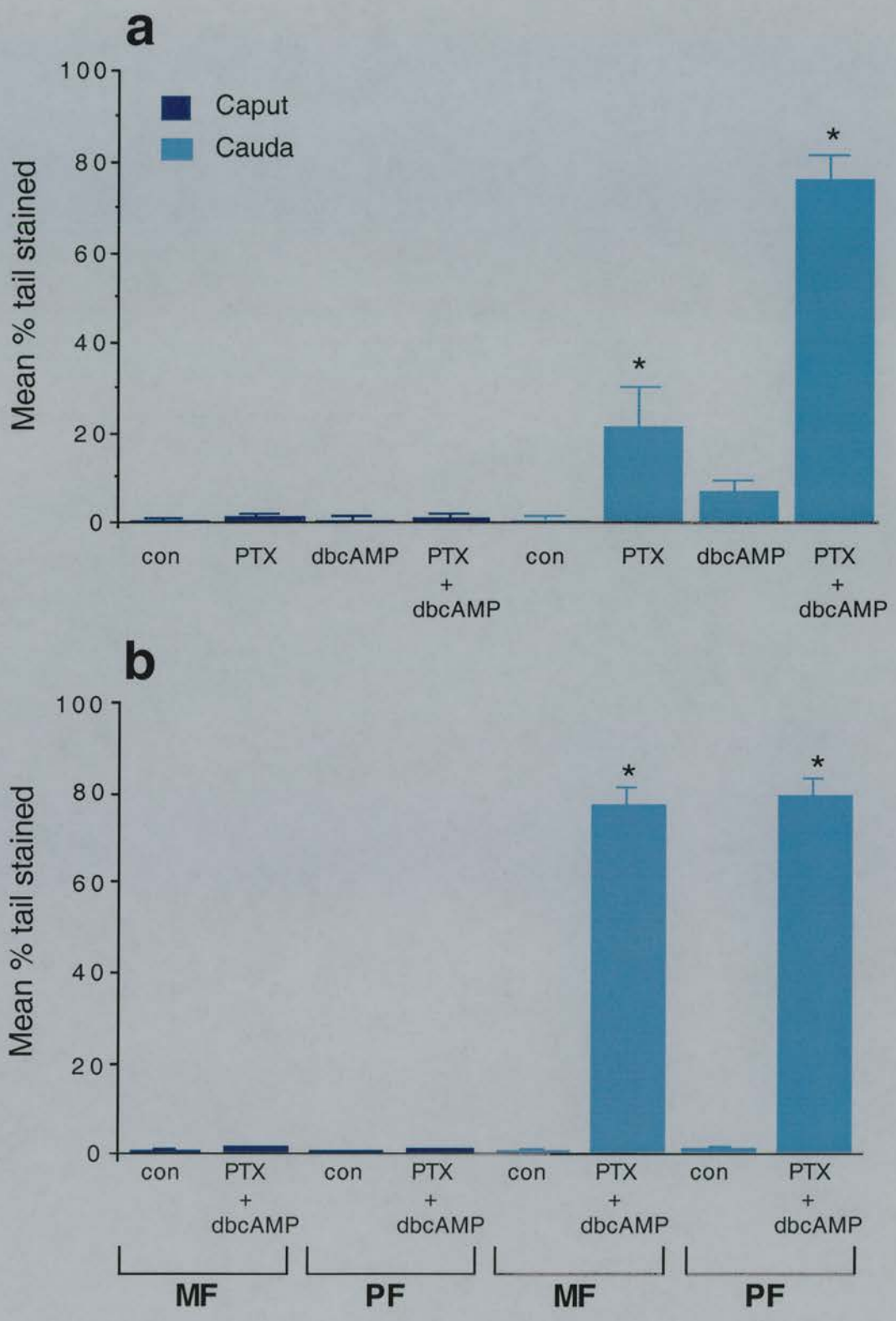

d
100
80
60
40
20
0
Mean % stained in entire acrosomal domain
3 mM
1 mM
500 μM
PTX concentration

Figure 4.6. **(a)** Graph representing the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation in the tail, following incubation with 3mM PTX and 5mM dbcAMP in BWW at 37°C for 3 hours (methanol fixed) (n = 3). **(b)** Comparison of tail phosphorylation in methanol fixed (MF) and paraformaldehyde fixed (PF) caput and caudal spermatozoa, following incubation with 3mM PTX and 5mM dbcAMP in BWW at 37°C for 3 hours (n = 3).


a
Caput
Cauda
Mean % tail stained
100
80
60
40
20
0
*
*
con
PTX
dbcAMP
PTX + dbcAMP
con
PTX
dbcAMP
PTX + dbcAMP
b
Mean % tail stained
100
80
60
40
20
0
*
*
con
PTX + dbcAMP
con
PTX + dbcAMP
con
PTX + dbcAMP
con
PTX + dbcAMP
MF
PF
MF
PF

4.3.iii The effect of PTX on intracellular cAMP levels in rat epididymal spermatozoa

As previously demonstrated in Chapter three, spermatozoa from the different regions of the epididymis are capable of generating equal levels of cAMP. There was no statistically significant difference in intracellular cAMP concentration between caput and caudal spermatozoa, although levels tended to be higher in caput cells. In addition, levels of PKA appeared to be the same in both the immature caput and mature caudal spermatozoa. Consequently it was of interest to determine whether or not there were any differences in phosphodiesterase activity, as demonstrated by the ability of PTX to increase intracellular cAMP levels.

When cells were stimulated with 3 mM PTX, intracellular cAMP levels were increased in both the immature caput and mature caudal spermatozoa, although the levels achieved were not statistically significant (Fig. 4.7.). Such results could explain why the addition of dbcAMP was also required to raise intracellular cAMP to the point where levels of tyrosine phosphorylation were maximised. These results also indicated that the distinct phosphorylation patterns observed in caput and caudal epididymal spermatozoa *in vivo* and *in vitro* were unlikely to be due to differences in intracellular cAMP alone.

4.3.iv PKA inhibition: The effect of PKA inhibitor H89 on tyrosine phosphorylation

In order to confirm PKA involvement in the cAMP mediated induction of tyrosine phosphorylation, rat spermatozoa were incubated in BWW supplemented with the PKA inhibitor H89 (10 μM), for 3 hours at 37°C, with and without 3 mM PTX and 5 mM dbcAMP. H89 was found to completely inhibit the PTX and dbcAMP associated inducement of tyrosine phosphorylation in both the immature caput and mature caudal rat epididymal spermatozoa (Fig. 4.8.).

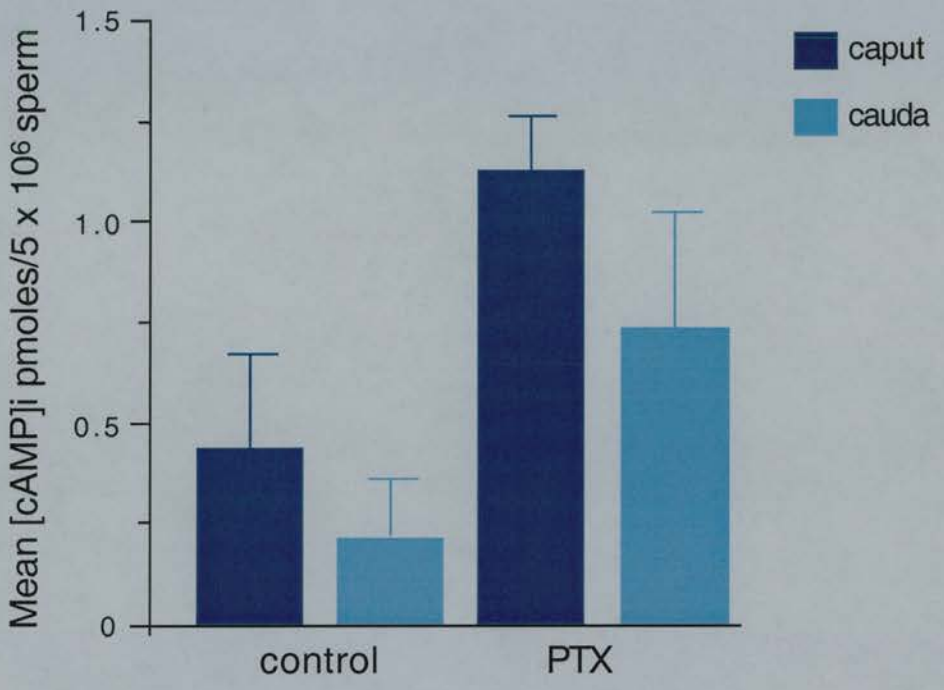
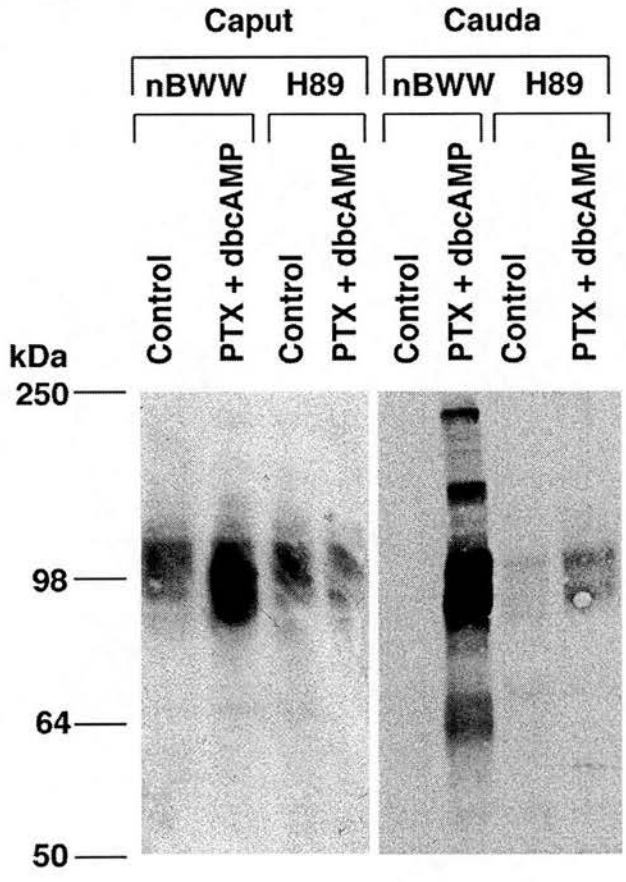


Figure 4.7. Data representing the mean intracellular concentration of cAMP in caput and caudal spermatozoa incubated for 3 hours in BWW with and without 3 mM PTX at 37°C (n = 6).

Figure 4.8. Western Blots representing the inhibition of tyrosine phosphorylation in rat spermatozoa by the PKA inhibitor, H89. Spermatozoa were incubated for 3 hours at 37°C with 3 mM PTX and 5 mM dbcAMP, in either nBWW or BWW supplemented with 10 μM H89. The sperm proteins were extracted with 1% SDS and separated by SDS-PAGE followed by Western Blot analysis with PY20, a monoclonal antibody against phosphotyrosine (n = 4).


Caput
Cauda
nBWW
H89
nBWW
H89
Control
PTX + dbcAMP
Control
PTX + dbcAMP
Control
PTX + dbcAMP
Control
PTX + dbcAMP
kDa
250
98
64
50

4.3.v Tyrosine kinase inhibition: The effect of tyrosine kinase inhibitor genistein on tyrosine phosphorylation

Tyrosine kinase involvement in the cAMP mediated effects on phosphotyrosine expression in the sperm tail was indicated by the ability of genistein to significantly suppress this response (Fig. 4.9. and 4.10.). Genistein was found to significantly reduce the proportion of caudal spermatozoa exhibiting phosphorylation of the tail, following incubation with 3 mM PTX and 5 mM dbcAMP (Fig. 4.10b.). This reduction in phosphorylation was confirmed by Western Blot analysis (Fig. 4.9.).

Figure 4.9. Western Blots representing rat sperm proteins extracted with 1% SDS and probed with an antibody against phosphotyrosine (PY20). The spermatozoa had been incubated in either **(a)** normal BWW or **(b)** BWW supplemented with 10 μM genistein for 3 hours at 37°C: **1** = control, **2** = 3 mM PTX + 5 mM dbcAMP (n = 4).

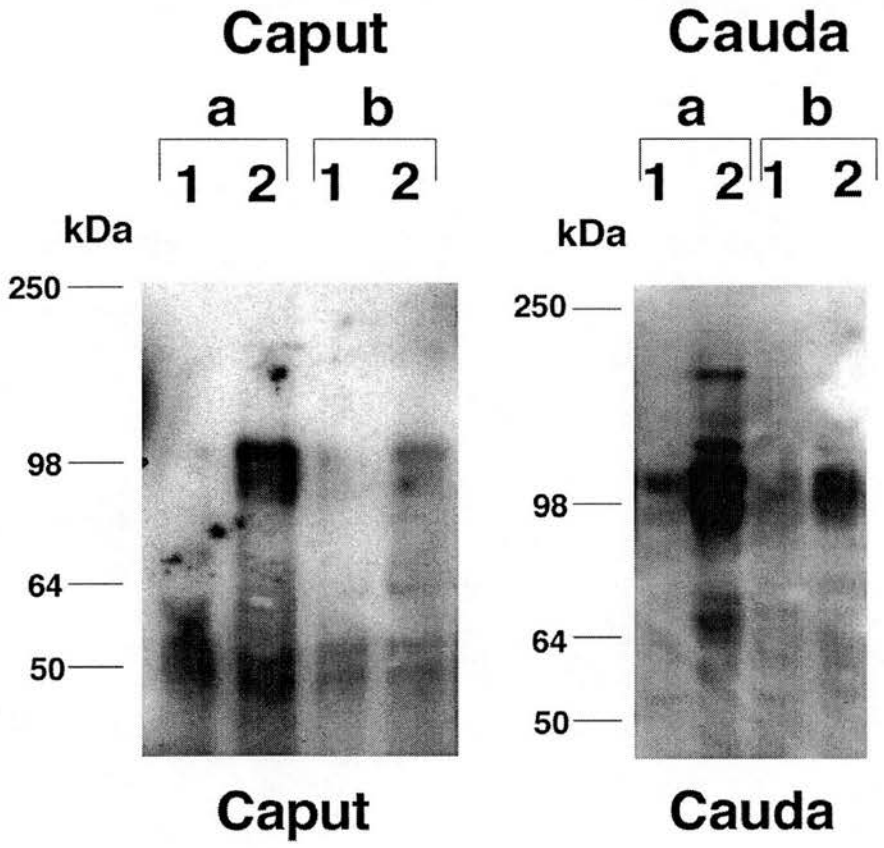
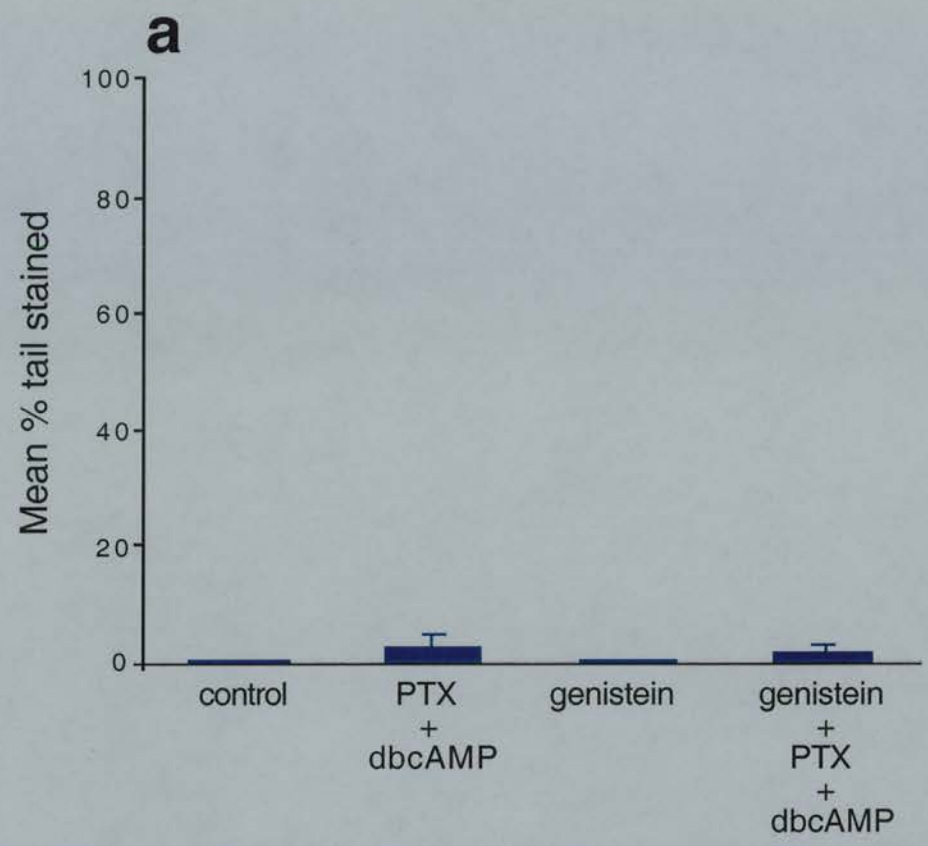
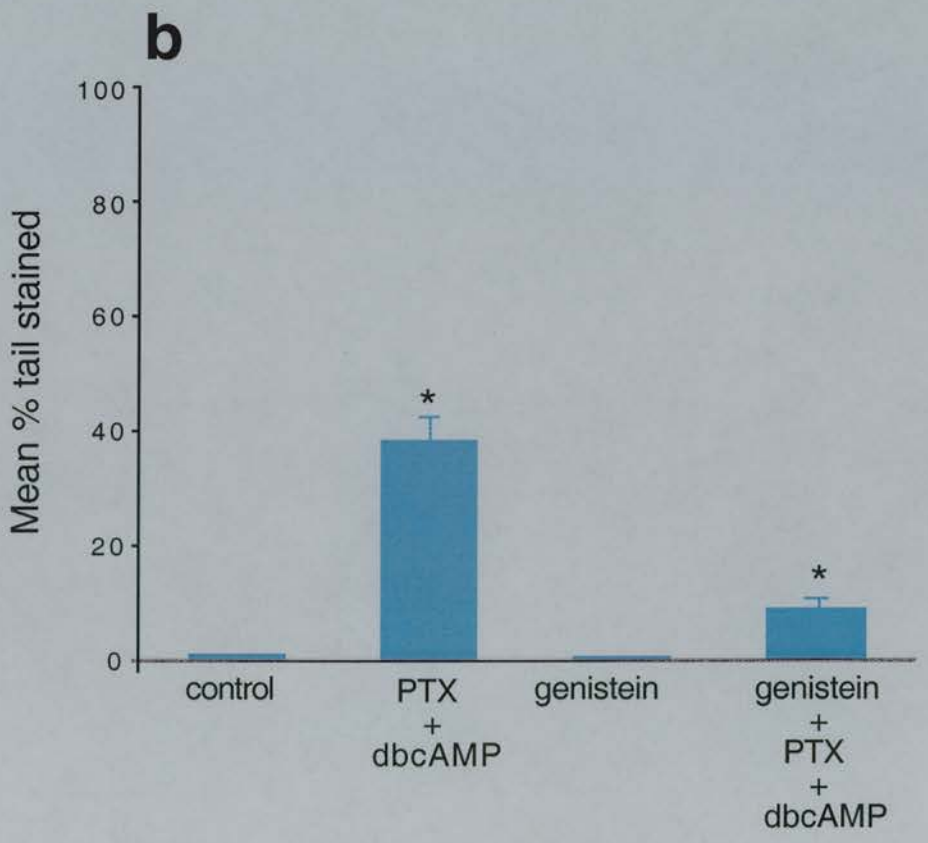

Caput
a
b
1 2 1 2
kDa
250
98
64
50
Caput
Cauda
a
b
1 2 1 2
kDa
250
98
64
50
Cauda

Figure 4.10. Graph representing the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation, following incubation with 10 μM genistein (n = 3).
(a) Percentage of caput spermatozoa exhibiting tyrosine phosphorylated proteins in the tail following 3 hours incubation with various treatments in BWW at 37°C. **(b)** Percentage of caudal spermatozoa exhibiting tyrosine phosphorylated proteins in the tail following 3 hours incubation with various treatments in BWW at 37°C.


a
Mean % tail stained
100
80
60
40
20
0
control
PTX + dbcAMP
genistein
genistein + PTX + dbcAMP


b
Mean % tail stained
100
80
60
40
20
0
*
*
control
PTX + dbcAMP
genistein
genistein + PTX + dbcAMP

4.4. Discussion

In accordance with many other species including sea urchin (Bookbinder *et al.*, 1991), Cynomologous monkey (Mahoney and Gwathmey, 1999), human (Aitken *et al.*, 1998a; Leclerc *et al.*, 1996), bovine (Brandt and Hoskins, 1980; Vijayaraghavan *et al.*, 1997a), boar (Kalab *et al.*, 1998) and the mouse (Visconti *et al.*, 1995b), we found that tyrosine phosphorylation in rat spermatozoa was also regulated by a cAMP-dependent signal transduction pathway. A particularly detailed and lucid study of this area has been conducted by Visconti *et al.*, 1995b, who found that active cAMP analogues could induce phosphorylation of capacitation-associated proteins in mature caudal mouse spermatozoa. Similarly, we found many new proteins were phosphorylated in mature caudal rat spermatozoa following the addition of the active cAMP analogue, dbcAMP, and the phosphodiesterase inhibitor, PTX, in a dose dependent manner. Western Blot analysis demonstrated that tyrosine phosphorylation was most pronounced when PTX and dbcAMP were used in combination, suggesting that these cells possess a particularly powerful phosphodiesterase. Such activity may contribute significantly to the low levels of tyrosine phosphorylation observed in untreated caudal epididymal rat spermatozoa and the difficulties encountered in capacitating these cells *in vitro*. Further evidence suggesting the presence of potent phosphodiesterases was provided by work carried out in the mouse (Visconti *et al.*, 1995b). In addition to a cAMP analogue, the presence of the phosphodiesterase inhibitor IBMX was required in order to fully restore the capacitation-associated phosphorylation of mouse caudal spermatozoa when incubated in media devoid of bicarbonate, calcium chloride or bovine serum albumin (Visconti *et al.*, 1995b).

PTX and dbcAMP also induced an increase in protein tyrosine phosphorylation in spermatozoa retrieved from the caput epididymis. However, this stimulatory effect was small compared with the dramatic stimulation of tyrosine expression seen in caudal sperm. Moreover it involved a completely different group of proteins, located in a completely different region of the cell. The stimulation of caudal spermatozoa with PTX and dbcAMP, induced the phosphorylation of a new set of proteins in the sperm tail while caput spermatozoa treated under the same conditions, only exhibited phosphotyrosine expression in the acrosomal region of the sperm head. The failure of caput epididymal spermatozoa to respond to cAMP with tyrosine phosphorylation in the sperm tail could reflect particularly high levels of phosphatase activity in the flagella apparatus of immature cells. Alternatively the observed differences could be due to the lack of appropriate protein kinase activity, although this is a less likely hypothesis as phosphotyrosine expression is high in caput spermatozoa, the only

difference being its confinement to the acrosomal region of the head. Consequently protein kinases must be active in order to explain the phosphorylations observed in both untreated and PTX/dbcAMP treated immature caput spermatozoa.

Immunocytochemical analysis also indicated the presence of highly active phosphodiesterases, as the percentage population of caudal spermatozoa exhibiting phosphorylation of the flagellum was significantly greater in those cells treated with the combined treatment of PTX and dbcAMP, than spermatozoa treated with dbcAMP alone. In addition, a significantly greater proportion of caudal spermatozoa exhibited the characteristic tail phosphorylations following incubation with PTX, in comparison with those treated with dbcAMP. Treatment of caudal spermatozoa with dbcAMP also failed to significantly increase the proportion of cells demonstrating phosphorylation of the tail, when compared with the untreated population of spermatozoa. Together these findings provide further evidence to support the hypothesis that highly potent phosphodiesterases are present in rat epididymal spermatozoa.

However, PTX treatment alone was insufficient to induce tyrosine phosphorylation of equal magnitude to that induced by the combined treatment of PTX and dbcAMP, as exemplified by Western Blot and immunocytochemical analysis. This indicates that PTX did not stimulate intracellular cAMP to levels sufficient to drive the tyrosine phosphorylation signal transduction cascade to maximal capacity. Further evidence for this suggestion is provided by the observation that increases in intracellular cAMP induced by PTX were not statistically significant, when compared to unstimulated intracellular levels, in either caput or caudal epididymal rat spermatozoa.

As described in Chapter Three, in terms of the cAMP/PKA kinase pathway, there appeared to be no significant differences between caput and caudal epididymal spermatozoa in the availability of intracellular cAMP as exemplified by their ability to respond to PTX stimulation in relation to their intracellular cAMP levels. The involvement of PKA in the regulation of tyrosine phosphorylation in rat spermatozoa was clarified by the ability of PKA inhibitor, H89 to suppress tyrosine phosphorylation in both caput and caudal epididymal spermatozoa. Clearly the PKA present in mammalian spermatozoa is actively engaged in regulating the tyrosine phosphorylation status of these cells. As demonstrated here, the PKA inhibitor, H89, has the ability to inhibit phosphorylation in rat spermatozoa, in addition to observations in the human (Aitken *et al.*, 1998a) and mouse (Visconti *et al.*, 1995b). If the cAMP/PKA pathway is functional at all stages of epididymal maturation it suggests that the changes observed in this study are likely to be due to differences in phosphatase activity.

The ability of the tyrosine kinase inhibitor genistein, to suppress significantly, tyrosine phosphorylation levels in both caput and caudal spermatozoa as demonstrated by Western Blot analysis, confirms the involvement of tyrosine kinase activity in the regulation of this signal transduction pathway. These observations eradicate further, the possibility that the inability of caput spermatozoa to demonstrate flagella phosphorylation in response to increased intracellular cAMP, is due to suppressed tyrosine kinase activity. These results clearly indicate functional tyrosine kinase activity as exemplified by the genistein induced inhibition of tyrosine phosphorylation. However, tyrosine kinase inhibition in immature caput spermatozoa cannot be dismissed completely as many types of tyrosine kinase exist and it is possible that the type located in the flagella of the spermatozoon is inactive in caput spermatozoa.

Genistein was effective in suppressing the *de novo* phosphorylations induced in the tail of caudal cells by a combination of PTX and dbcAMP. Although genistein suppressed tyrosine phosphorylation in caput spermatozoa as observed by Western Blot analysis, no significant effect could be observed on the localisation of phosphorylation in these cells. However, this could be due to the fact that the immunocytochemical analysis used in this study provides a method of measuring the cell population exhibiting a specific pattern of staining but it does not take into account the intensity of the positive stain. Consequently inhibition of phosphotyrosine expression may fail to be detected by this method of evaluation. Similarly, up-regulation in tyrosine phosphorylation may also be difficult to localise by this form of analysis, if the newly phosphorylated proteins are in the same location as the phosphoproteins already present, as evident in the spermatozoa obtained from the caput epididymis. In contrast, changes in caudal spermatozoa were easily detected by this method as genistein inhibited the PTX and dbcAMP-associated induction of phosphorylation of the tail, which was visually evident. These results may be correlated with what has been demonstrated in the human, whereby capacitation-associated tyrosine phosphorylation was also found to be genistein-sensitive (Carrera *et al.*, 1996).

In conclusion, the results presented in this chapter indicate that epididymal maturation of rat spermatozoa is associated with an acquired competence to respond to high levels of intracellular cAMP by phosphorylating tyrosine residues on the sperm tail. The inability of immature spermatozoa situated in the caput epididymis to respond to stimulation of intracellular cAMP levels is unlikely to be due to inhibition of tyrosine kinase activity or reduced availability of cAMP. A possible explanation is the increased activity of phosphatases that may cause the dephosphorylation of target proteins in these immature cells, and this is explored further in Chapter Seven.

Chapter Five:

The Generation of Reactive Oxygen Species (ROS) by Rat Epididymal Spermatozoa and their Function in Relation to Tyrosine Phosphorylation

Chapter 5. The Generation of Reactive Oxygen Species (ROS) by Rat Epididymal Spermatozoa and their Function in Relation to Tyrosine Phosphorylation

5.1. Introduction

An atom or group of atoms possessing an odd (unpaired) electron is called a free radical and they are extremely reactive due to their desire to gain another electron (Morrison and Boyd, 1992). The reactivity of these molecules results from the fact that more energy is required for the maintenance of two separate species, each with an unpaired electron, than for them to come together and share electrons, such that a full molecular orbital is established along with a covalent bond (Aitken and Fisher, 1994).

Reactive oxygen species (ROS) belong to the category of free radicals and they include the following species: the superoxide anion ($O_2^{\cdot-}$), the hydroperoxyl radical ($HO_2^{\cdot}$) and non-radical derivatives also grouped under this collective term of biological relevance include hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Aitken and Fisher, 1994).

ROS are generally associated with phagocytes that utilise these extremely reactive species during the 'respiratory burst' that mediates the destruction of invading cells and organisms (Babior *et al.*, 1973). However, the ability of many non-phagocytic cells to generate ROS has been identified in such cell types as endothelial cells, mesangial cells, fibroblasts, thyroid cells, Leydig cells, Epstein-Barr transformed human B lymphocytes, adipocytes, tumour cells and oocytes (Cross and Jones, 1991; Dupuy *et al.*, 1989; Meier *et al.*, 1991). In addition to the potential destruction of the respiratory burst, these molecules have been shown to play a positive functional role in the electron transfer reactions involved in cell regulation (Cross and Jones, 1991).

The generation of ROS by mammalian spermatozoa was first described in 1943 by an andrologist called John MacLeod who demonstrated that human spermatozoa were capable of generating H_2O_2 (Macleod, 1943). Three years later, Tosic and Walton observed that bull spermatozoa also possessed the ability to generate H_2O_2 (Tosic and Walton, 1946). Subsequently, data describing the production of H_2O_2 by mouse (Alvarez and Storey, 1984) and rabbit (Alvarez and Storey, 1982) spermatozoa has been published. The spontaneous generation of $O_2^{\cdot-}$ and its subsequent dismutation

into H_2O_2, by the action of an intracellular supply of superoxide dismutase (SOD) has also been established in human spermatozoa (Aitken and Clarkson, 1987a; Aitken and Clarkson, 1987b; Alvarez *et al.*, 1987).

Much information has been gathered concerning the role of ROS in the regulation of tyrosine phosphorylation in disparate cell types including spermatozoa. Autophosphorylation of the β-subunit of the insulin receptor can be induced following exposure to H_2O_2 (Koshio *et al.*, 1988; Mukherjee *et al.*, 1978) and H_2O_2 has been shown to act synergistically with vanadate to induce tyrosine phosphorylation in human peripheral blood T cells (O'Shea *et al.*, 1992). Hydrogen peroxide has also been shown to inactivate a low molecular weight phosphotyrosine-protein phosphatase (Caselli *et al.*, 1998). The generation of ROS by human spermatozoa plays a key role in the control of sperm function via the redox regulation of tyrosine phosphorylation (Aitken *et al.*, 1995) and the available data suggests that human spermatozoa possess an NADPH oxidase system for the generation of these species (Aitken *et al.*, 1997). Results presented by Zhang and Zheng (Zhang and Zheng, 1996) indicated that only capacitated human spermatozoa were able to generate $O_2^{\cdot-}$ and this in turn stimulated capacitation further, probably due to its positive effect on intracellular cAMP levels.

Helen Fisher previously demonstrated in our laboratory the ability of guinea pig, mouse, hamster and rat epididymal spermatozoa to generate ROS (Fisher and Aitken, 1997). Consequently, it was a natural progression to extend these findings by investigating the impact of ROS on tyrosine phosphorylation in rat spermatozoa, with the aim of determining if indeed this process was redox-regulated, and how it developed during epididymal maturation in concert with the evolving competence of spermatozoa to undergo capacitation.

5.2. Materials and Methods

Refer to Chapter 2 for general materials and methods.

5.2.i. Spontaneous superoxide ($O_2^{\cdot-}$) generation in epididymal rat spermatozoa

As detailed in Section 2.6., detection of $O_2^{\cdot-}$ was measured by lucigenin-dependent chemiluminescence using a Berthold luminometer (LB9505, Berthold Analytical Instruments, Wildbad, Germany) at 37°C. Lucigenin is a charged compound used for the detection of superoxide. It is therefore relatively membrane impermeant and thus largely detects the extracellular release of $O_2^{\cdot-}$. Rat spermatozoa were extracted from the epididymis as described in Section 2.3., motility and density counts (Sections 2.4. and 2.5. respectively) were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The procedure for detection of spontaneous $O_2^{\cdot-}$ generation by rat epididymal spermatozoa was carried out exactly as described in Section 2.6.i.

5.2.ii. Spontaneous hydrogen peroxide (H_2O_2) generation in epididymal rat spermatozoa

As detailed in Section 2.6., detection of $O_2^{\cdot-}$ was measured by luminol-dependent chemiluminescence using a Berthold luminometer (LB9505, Berthold Analytical Instruments, Wildbad, Germany) at 37°C. Luminol, when used alongside HRP, can measure the production of hydrogen peroxide intra- and extracellularly. Rat spermatozoa were extracted from the epididymis as described in Section 2.3., motility and density counts (Sections 2.4. and 2.5. respectively) were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The procedure for detection of spontaneous H_2O_2 generation by rat epididymal spermatozoa was carried out exactly as described in Section 2.6.ii.

5.2.iii NADPH versus NADH induced ($O_2^{\cdot-}$) generation in epididymal rat spermatozoa

Rat spermatozoa were extracted from the epididymis as described in Section 2.3. and following motility and density counts (Sections 2.4. and 2.5. respectively), the sperm samples were adjusted to concentrations of 10 x 10^6/ml. Luminometer runs were set up in accordance with the criteria referred to in Section 2.6.i. When the addition of either NADPH or NADH was required, the luminometer run was temporarily halted and NADPH/NADH diluted in BWW was added to the appropriate cuvettes, at a final concentration of 2 mM. Immediately following the addition of NADPH/NADH, the luminometer run was restarted.

5.2.iv The effect of (a) NADPH/NADH and (b) tyrosine phosphatase inhibitor, zinc chloride on tyrosine phosphorylation in epididymal rat spermatozoa

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one caput epididymis was diluted into normal BWW while those from the other were diluted straight into BWW that had been supplemented with 100 µM zinc chloride. The same procedure was then carried out on the spermatozoa from the caudal region. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.). The spermatozoa were incubated with various treatments including 3 mM PTX, 2 mM NADPH and 2 mM NADH for 3 hours at 37°C. On completion of the three hour incubation period, rat sperm proteins were extracted using SDS as described in Section 2.7. and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11. In addition, slides were prepared from some of the sperm samples as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of tyrosine phosphorylated proteins. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14.

5.2.v The effect of NADPH and zinc chloride on intracellular cAMP levels in epididymal rat spermatozoa

Following the release of rat spermatozoa into both normal BWW and BWW supplemented with 100 μM zinc chloride as described above in Section 5.2.iv., motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. Spermatozoa were incubated at this concentration with either 3 mM PTX or the combined treatment of 3 mM PTX and 2 mM NADPH for 3 hours at 37°C. Following completion of the incubation period, extraction of cAMP from the spermatozoa was performed as described in Section 2.15.

5.2.vi NADPH versus dbcAMP: Comparison of their tyrosine phosphorylation inducing capabilities

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and diluted in BWW at a concentration of 10 x 10^6/ml (Sections 2.4. and 2.5.). The spermatozoa were incubated with various treatments including 3 mM PTX, 2 mM NADPH and 5 mM dbcAMP for 3 hours at 37°C.

On completion of the incubation period, slides were prepared from the spermatozoa as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of tyrosine phosphorylated proteins. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14.

The remaining sperm were processed for Western Blot analysis as described in Sections 2.7.-2.11.

5.2.vii The effect of H_2O_2 and catalase on tyrosine phosphorylation in epididymal rat spermatozoa

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and diluted in BWW at a concentration of 10 x 10^6/ml (Sections 2.4. and 2.5.). The spermatozoa were incubated with 100 μM H_2O_2 or 5000 U catalase/ml of sperm in addition to the combined treatment of these two reagents for 3 hours at 37°C. Following the incubation period slides were set up for immunocytochemistry and fixed with paraformaldehyde as described in Section 2.14. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14. The remaining sperm were processed for Western Blot analysis as described in Sections 2.7.-2.11.

5.2.viii The effect of H_2O_2 on intracellular cAMP levels in epididymal rat spermatozoa

Following the release of rat spermatozoa into either normal BWW or BWW supplemented with 100 μM H_2O_2 as described in Section 2.3., motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. The spermatozoa were incubated at this concentration with either 3 mM PTX or the combined treatment of 3 mM PTX and 2 mM NADPH for 3 hours at 37°C. Following completion of the incubation period, extraction of cAMP from the spermatozoa was performed as described in Section 2.15.

5.3 Results

5.3.i. Spontaneous superoxide ($O_2^{\cdot-}$) generation in epididymal rat spermatozoa

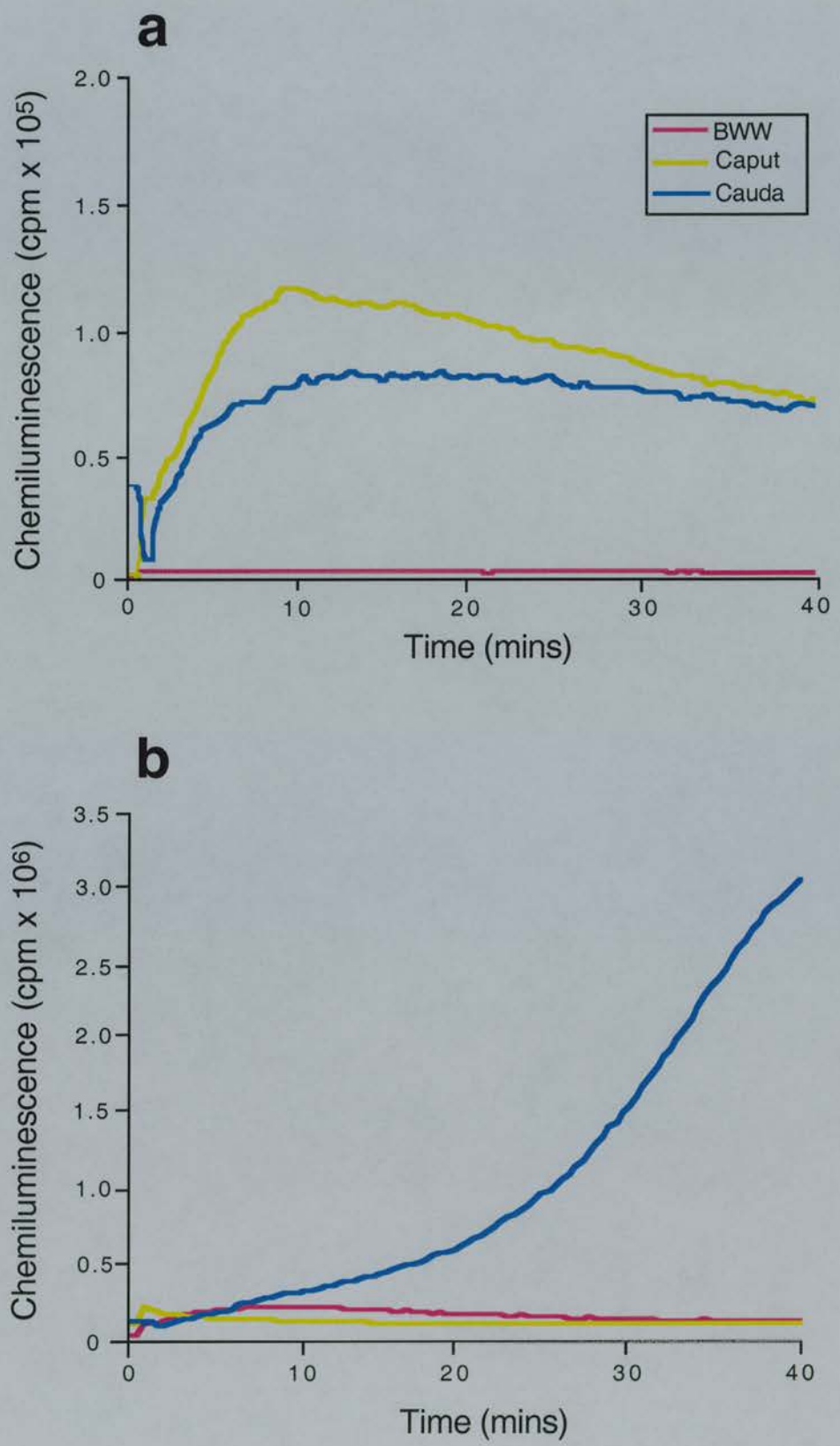
The spontaneous generation of reactive oxygen species (ROS) in a mammalian species, was first demonstrated in human spermatozoa (Macleod, 1943) followed by the bull (Tosic and Walton, 1946). The generation of ROS has subsequently been identified further in a wide range of species including rabbit (Holland and Storey, 1981), rat, guinea pig, mouse, hamster (Fisher and Aitken, 1997) and human (Aitken and Clarkson, 1987a; Aitken and Clarkson, 1987b). It has since been established that capacitation is a redox regulated event during which oxidising conditions enhance the attainment of a capacitated state through the induction of tyrosine phosphorylation (Aitken *et al.*, 1995).

Thus it was of interest to try and determine if similar redox regulating mechanisms existed in rat spermatozoa. Lucigenin-dependent chemiluminescence demonstrated that both caput and caudal spermatozoa spontaneously generate $O_2^{\cdot-}$ (Fig. 5.1a.). Initially caput spermatozoa appeared to generate more $O_2^{\cdot-}$ than those extracted from the cauda, however, during the course of a 40 minute incubation the levels of spontaneous generated $O_2^{\cdot-}$ by the caput cells levelled out to approximately equal production by caudal spermatozoa.

5.3.ii. Spontaneous hydrogen peroxide (H_2O_2) generation in epididymal rat spermatozoa

In contrast to $O_2^{\cdot-}$ generation, spontaneous H_2O_2 production by the two types of cells differed considerably. Luminol-dependent chemiluminescence demonstrated that caput cells produced very low levels of H_2O_2 whereas caudal cells exhibited a continual increase in the amount of H_2O_2 over a period of 40 minutes (Fig. 5.1b).

Figure 5.1. Comparison between rat spermatozoa extracted from the caput and cauda epididymis in their ability to spontaneously generate $O_2^{\cdot-}$ and H_2O_2:
(a) Lucigenin-dependent chemiluminescence representing spontaneous $O_2^{\cdot-}$ generation in both caput and caudal spermatozoa. BWW media was used as a control (n = 6).
(b) Luminol/peroxidase-dependent chemiluminescence representing spontaneous H_2O_2 generation in both caput and caudal spermatozoa. BWW media was used as a control (n = 4).


a
Chemiluminescence (cpm x 10⁵)
2.0
1.5
1.0
0.5
0
BWW
Caput
Cauda
0
10
20
30
40
Time (mins)
b
Chemiluminescence (cpm x 10⁶)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
Time (mins)

5.3.iii NADPH versus NADH induced ($O_2^{-\cdot}$) generation in epididymal rat spermatozoa

It has been suggested that human spermatozoa possess an NADPH oxidase system for the generation of ROS (Aitken and Clarkson, 1987a; Aitken and Clarkson, 1987b) and in keeping with previous findings (Fisher and Aitken, 1997), $O_2^{-\cdot}$ production by rat spermatozoa was examined in the presence of 2 mM NADPH. Caput and caudal spermatozoa were shown to generate $O_2^{-\cdot}$ in response to exogenous NADPH. However, the response elicited in caudal cells was significantly reduced compared to that exhibited by caput spermatozoa (Fig. 5.2. and 5.3.).

NADH elicited a similar $O_2^{-\cdot}$ response in caput and caudal spermatozoa as that initiated by NADPH. The amount of $O_2^{-\cdot}$ produced by caput spermatozoa in response to NADH was significantly greater than the controls and mature caudal spermatozoa (Fig. 5.2. and 5.3.).

Statistical analysis demonstrated no significant difference between NADPH and NADH induced $O_2^{-\cdot}$ levels in either caput or caudal spermatozoa (Fig. 5.2. and 5.3.).

Figure 5.2. Comparison between rat spermatozoa extracted from the caput and cauda epididymis in their ability to generate $O_2^{-\cdot}$ in response to NADPH and NADH. Either 2mM NADH or NADPH was added to each sample including the BWW control (n = 5).

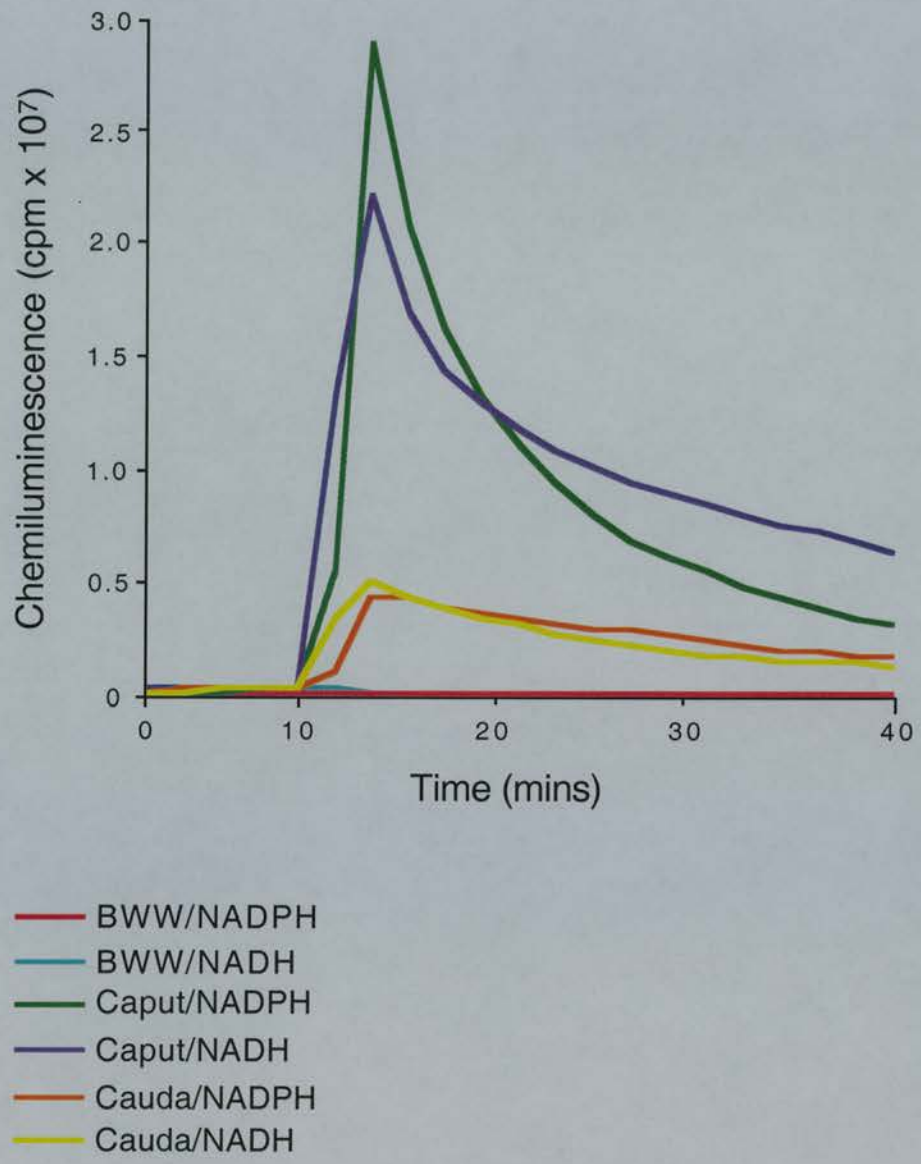
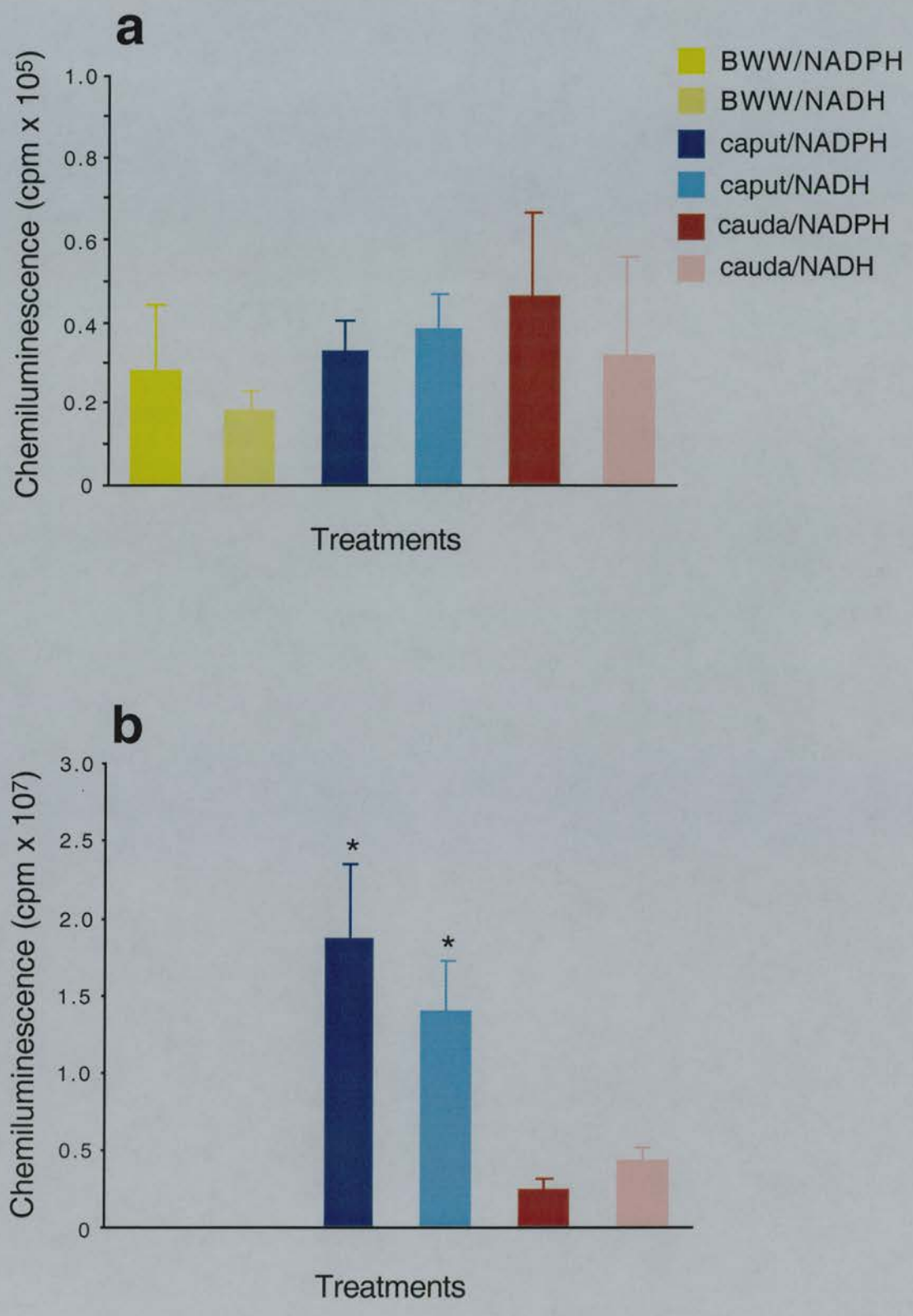
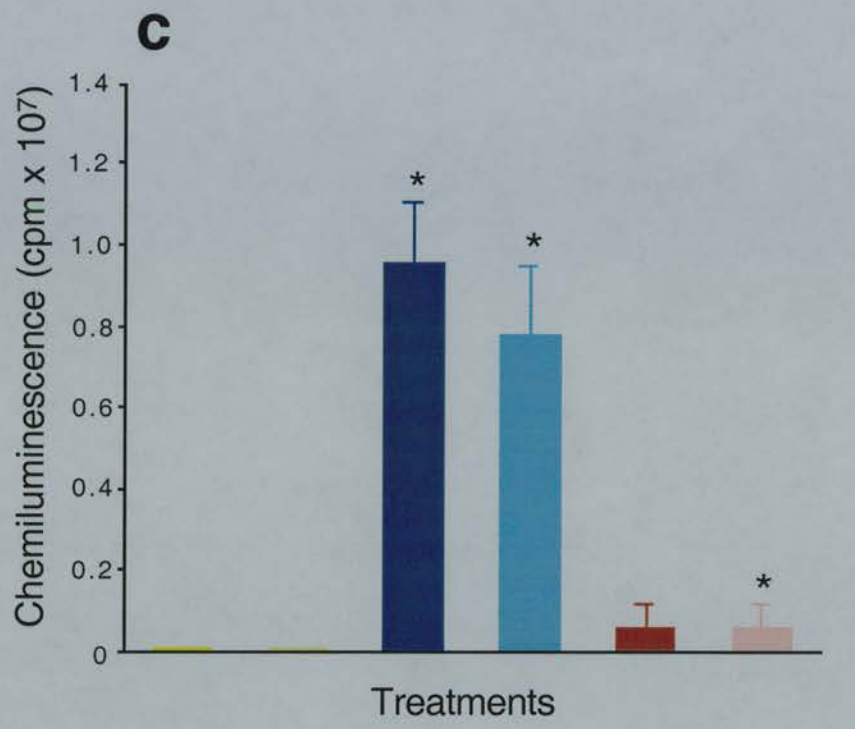
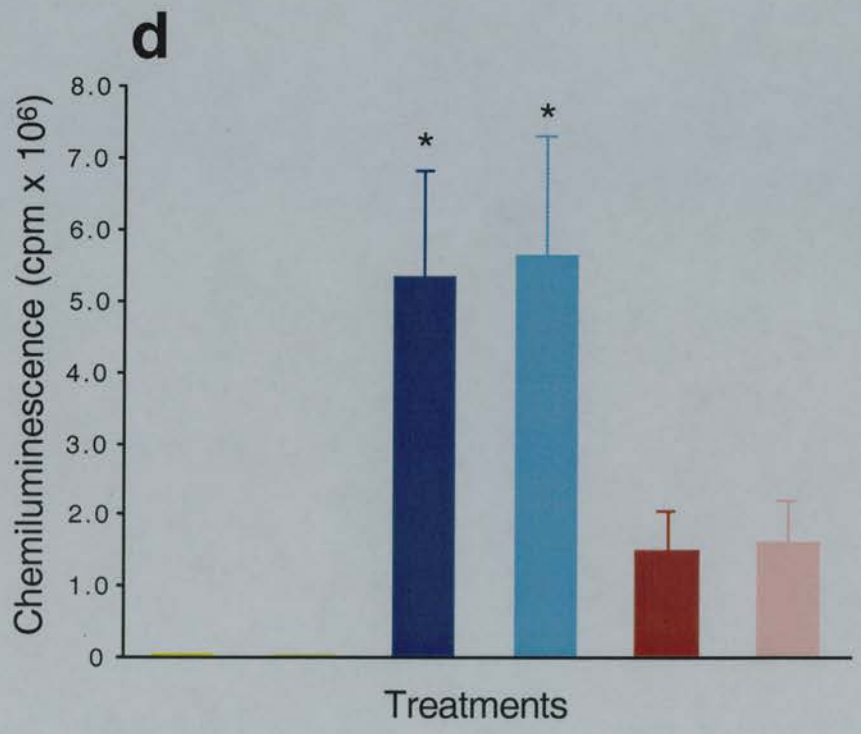
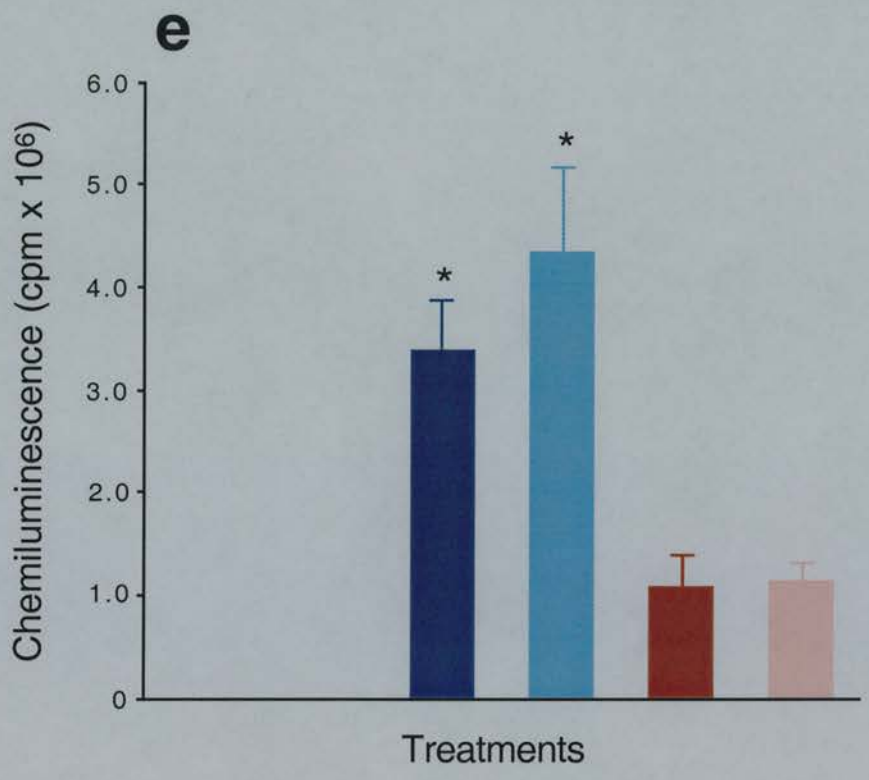

Chemiluminescence (cpm x 10⁷)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
Time (mins)
BWW/NADPH
BWW/NADH
Caput/NADPH
Caput/NADH
Cauda/NADPH
Cauda/NADH

Figure 5.3. The following bar charts represent the mean production of $O_2^{\cdot-}$ in response to NADPH and NADH in rat spermatozoa. Superoxide induced chemiluminescence was measured in counts per minute (cpm) following **(a)** 0 minutes, **(b)** 12 minutes, **(c)** 20 minutes, **(d)** 30 minutes and **(e)** 40 minutes incubation (n = 5).


a
Chemiluminescence (cpm x 10⁵)
1.0
0.8
0.6
0.4
0.2
0
BWW/NADPH
BWW/NADH
caput/NADPH
caput/NADH
cauda/NADPH
cauda/NADH
Treatments
b
Chemiluminescence (cpm x 10⁷)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
Treatments


c
Chemiluminescence (cpm x 10⁷)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*
Treatments


d
Chemiluminescence (cpm x 10⁶)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
*
*
Treatments


e
6.0
5.0
4.0
3.0
2.0
1.0
0
Chemiluminescence (cpm x 10⁶)
*
*
Treatments

5.3.iv The effect of (a) NADPH/NADH and (b) tyrosine phosphatase inhibitor, zinc chloride on tyrosine phosphorylation in epididymal rat spermatozoa

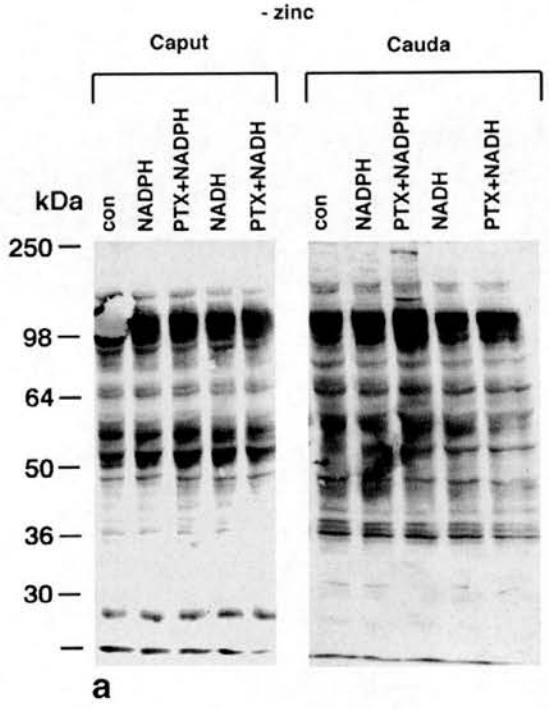
(a) When ROS production by caput and caudal cells was stimulated with NADPH (2 mM) for 3 hours, no increase in tyrosine phosphorylation was observed (Fig. 5.4a.). However, when rat spermatozoa were incubated with 2 mM NADPH in the presence of a phosphodiesterase inhibitor 3 mM PTX, tyrosine phosphorylation of several proteins was up-regulated in cauda spermatozoa only (Fig. 5.4a.). The phosphorylation of two novel proteins of approximately 125 and 225 kDa was particularly evident in caudal but not caput spermatozoa (Fig. 5.4a.). Moreover, the phosphorylation of these bands was not observed in the presence of PTX alone (Fig. 5.7b.), or with the combination of NADH and PTX, indicating that the effect was NADPH specific (Fig. 5.4a.). This was interesting as we have also demonstrated that 2 mM NADH induces levels of ROS in rat spermatozoa equal to that stimulated by NADPH (Fig. 5.2. and 5.3.). Immunocytochemistry also demonstrated that phosphorylation in the tail of caudal spermatozoa was induced following the treatment of PTX with NADPH (Fig. 5.5.).

Together, these observations support the hypothesis that spermatozoa possess an NADPH oxidase system involved in the regulation of tyrosine phosphorylation and consequently capacitation. Since the presence of a phosphodiesterase inhibitor was necessary to ensure the NADPH effect, these results suggest that the redox regulation of tyrosine phosphorylation in rat spermatozoa is mediated by cAMP, as recently suggested for human spermatozoa (Aitken *et al.*, 1998a).

(b) The effect of zinc chloride on tyrosine phosphorylation was dependent on the maturation status of the spermatozoa. Zinc chloride did not increase tyrosine phosphorylation in caput spermatozoa, regardless of the treatment and it either had no effect at all, or actually down-regulated phosphorylation (Fig. 5.4.). However, in caudal cells, the presence of zinc chloride, induced the phosphorylation of the 125 and 225 kDa proteins in all of the treatments, including the controls in some animals (Fig 5.4).

Immunocytochemical analysis demonstrated that in mature caudal spermatozoa only, a significant increase in the proportion of cells exhibiting positive tail staining in cells treated with PTX alone and PTX with NADPH as well as the control, in comparison with corresponding samples incubated in BWW without zinc was observed (Fig. 5.5.).

Figure 5.4. The influence of antioxidant and tyrosine phosphatase inhibitor, zinc chloride on tyrosine phosphorylation in rat epididymal spermatozoa.
(a) A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in BWW at 37°C, for 3 hours with the following treatments: 2 mM NADPH; 3 mM PTX + 2 mM NADPH; 2 mM NADH; 3 mM PTX + 2 mM NADH (n = 4).
(b) A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in BWW supplemented with 100 μM zinc chloride at 37°C, for 3 hours with the following treatments: 2 mM NADPH; 3 mM PTX + 2 mM NADPH; 2 mM NADH; 3 mM PTX + 2 mM NADH (n = 4).


- zinc
Caput
Cauda
kDa
con
NADPH
PTX+NADPH
NADH
PTX+NADH
250
98
64
50
36
30
a

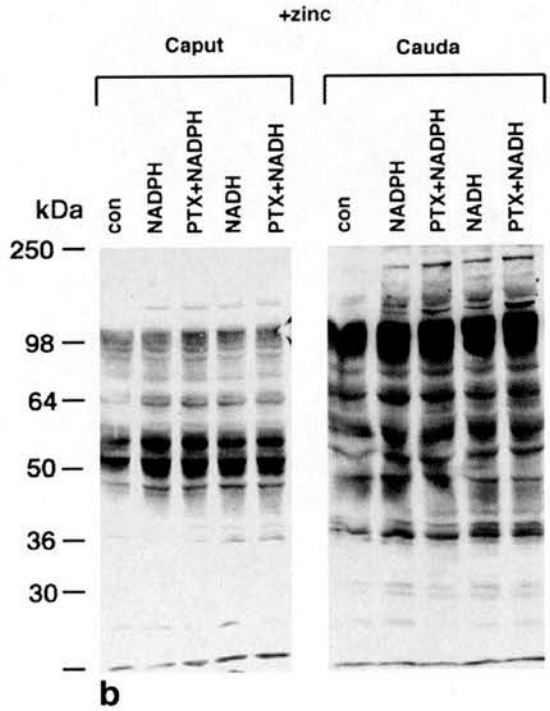
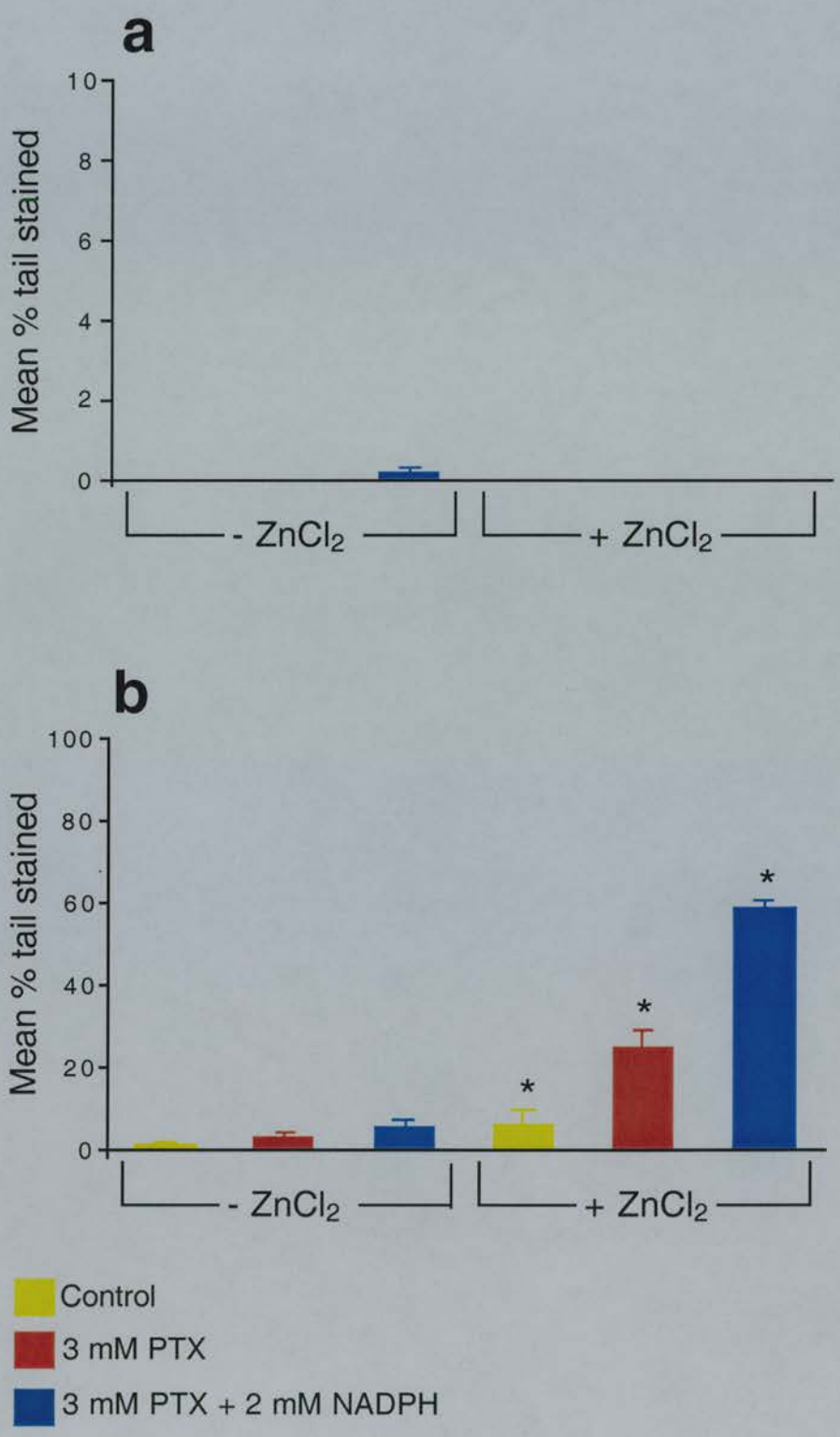

+zinc
Caput
Cauda
kDa
con
NADPH
PTX+NADPH
NADH
PTX+NADH
250
98
64
50
36
30
b

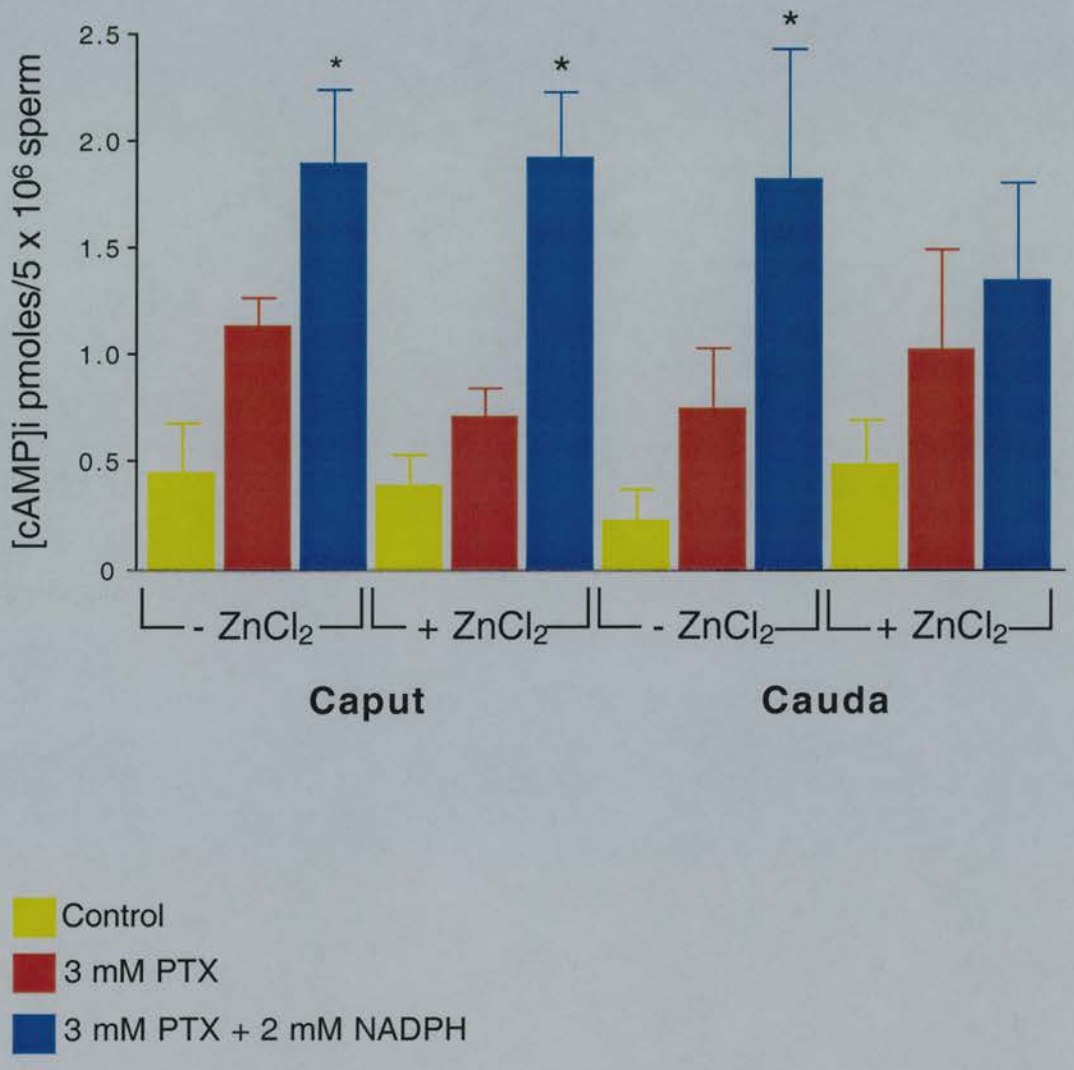
Figure 5.5. Graphical representation of the percentage population of **(a)** caput and **(b)** caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation in the tail, following incubation in either nBWW or BWW supplemented with 100 μM zinc chloride, at 37°C for 3 hours with the following treatments: 3 mM PTX; 3 mM PTX + 2 mM NADPH (n = 3).


a
Mean % tail stained
10
8
6
4
2
0
- ZnCl2
+ ZnCl2
b
Mean % tail stained
100
80
60
40
20
0
*
*
*
- ZnCl2
+ ZnCl2
Control
3 mM PTX
3 mM PTX + 2 mM NADPH

5.3.v The effect of NADPH on intracellular cAMP levels in epididymal rat spermatozoa

When sperm intracellular cAMP levels were evaluated, it was evident that [cAMP]i concentration in both caput and caudal spermatozoa was significantly raised following incubation for 3 hours at 37°C with 3 mM PTX and 2 mM NADPH. Moreover [cAMP]i levels following the combined treatment were significantly greater than when spermatozoa were stimulated with 3 mM PTX alone under the same conditions (Fig. 5.6.). Zinc chloride had no effect on intracellular cAMP levels (Fig. 5.6.).

Figure 5.6. Data representing the mean intracellular concentration of cAMP in caput and caudal spermatozoa incubated for 3 hours in either nBWW or BWW supplemented with 100 μM zinc chloride, at 37°C with the following treatments: 3 mM PTX; 3 mM PTX + 2 mM NADPH (n = 6).


[cAMP]i pmoles/5 x 10⁶ sperm
2.5
2.0
1.5
1.0
0.5
0
*
*
*
- ZnCl2
+ ZnCl2
- ZnCl2
+ ZnCl2
Caput
Cauda
Control
3 mM PTX
3 mM PTX + 2 mM NADPH

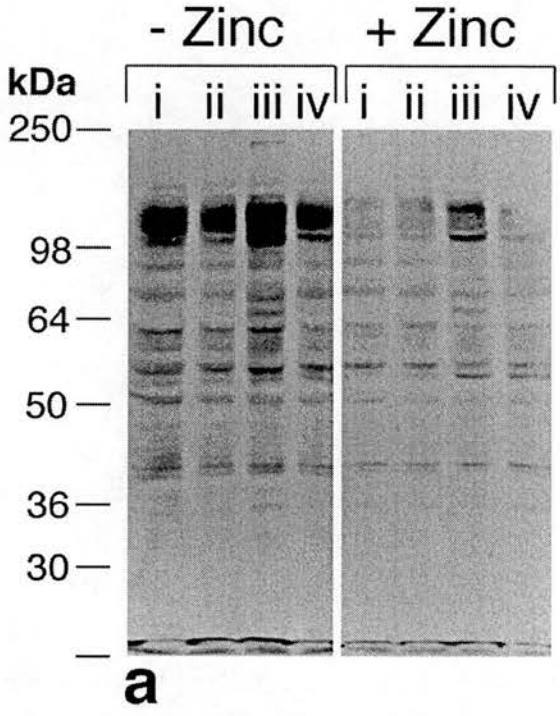
5.3.vi NADPH versus dbcAMP: Comparison of their tyrosine phosphorylation inducing capabilities

Interestingly, the 125 and 225 kDa proteins phosphorylated following stimulation with 2 mM NADPH in the presence of 3 mM PTX were also phosphorylated following incubation with PTX and dbcAMP (refer to Chapter 4). Immunocytochemistry demonstrated that phosphorylation in the tail of caudal spermatozoa was induced following the treatment of PTX with NADPH (Fig. 5.5. and 5.8.). This was in correlation with previous findings whereby treatment of caudal spermatozoa with 3 mM PTX and 5 mM dbcAMP resulted in tyrosine phosphorylation of tail proteins (refer to Chapter 4 and Fig. 5.8.).

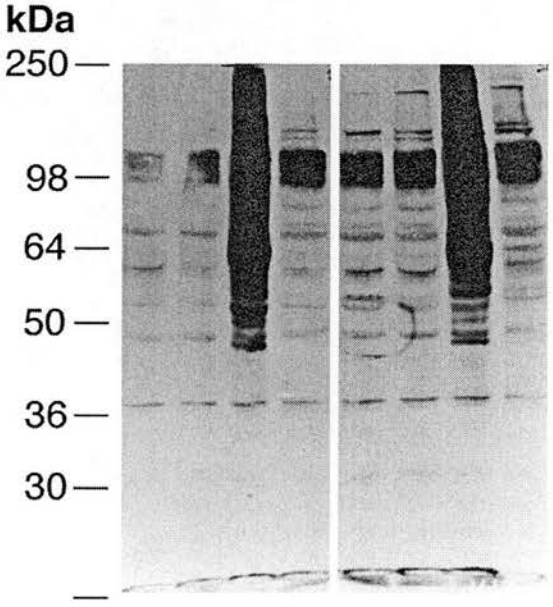
The fact that proteins of the same molecular weight (including those of 125 and 225 kDa) were phosphorylated at identical sites in the presence of either dbcAMP or NADPH suggests that redox-regulation of tyrosine phosphorylation in rat spermatozoa is mediated by cAMP. The main difference between the two treatments is that dbcAMP induced tyrosine phosphorylation of many proteins (Fig. 5.7.) whereas NADPH enhanced the phosphorylation of a smaller number of proteins, specifically those of approximately 125 and 225 kDa (Fig. 5.4.). Additionally, the percentage of the caudal sperm population exhibiting tail phosphorylation following the combined treatment of PTX with NADPH, was significantly lower (approximately one third of the population) in comparison with those treated with PTX and dbcAMP (Fig. 5.8.). However, lowered NADPH induced phosphorylation in comparison with that stimulated by dbcAMP is most probably quantitative rather than qualitative. 5 mM dbcAMP is likely to raise intracellular cAMP levels significantly more than 2 mM NADPH, as the generation of cAMP by NADPH is dependent on the rate of redox cycling, the synthetic capacity of adenylyl cyclase and the availability of ATP.

As demonstrated previously (Fig. 5.4.), incubation of rat spermatozoa in BWW supplemented with 100 μM zinc chloride lead to a reduction in tyrosine phosphorylation in those obtained from the caput epididymis, whereas an increase specifically in proteins of approximately 125 and 225 kDa, was observed in caudal spermatozoa (Fig. 5.7.).

Figure 5.7. Redox-regulation of tyrosine phosphorylation in rat spermatozoa. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from **(a)** caput and **(b)** caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either nBWW or BWW that had been supplemented with 100 μM zinc chloride at 37°C, for 3 hours with the following treatments: **(i)** control, **(ii)** 3 mM PTX, **(iii)** 3 mM PTX + 5mM dbcAMP, **(iv)** 3 mM PTX + 2 mM NADPH (n = 3).

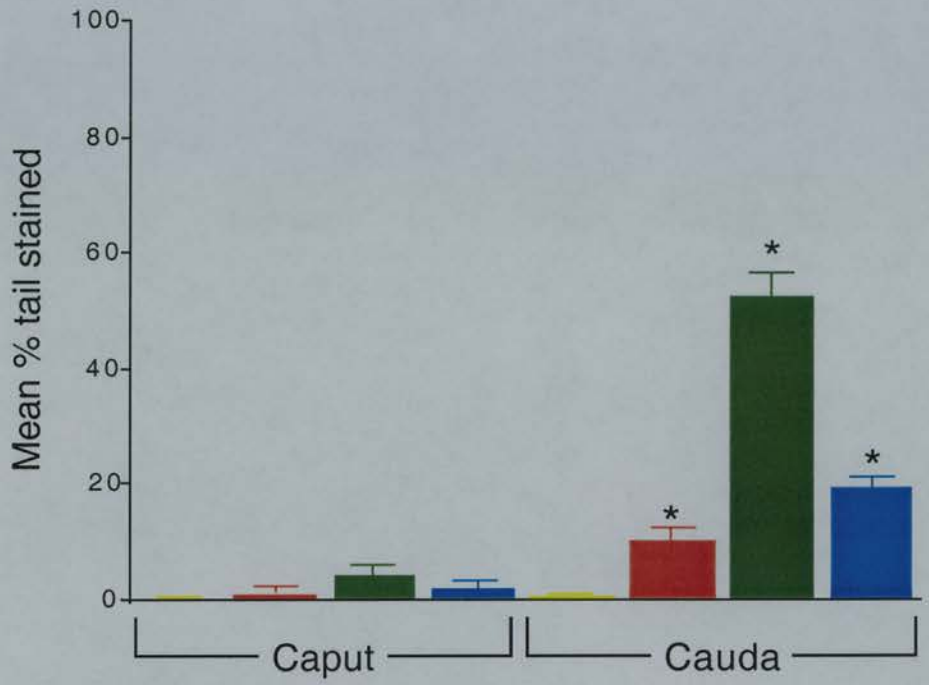

- Zinc
+ Zinc
kDa
i ii iii iv
i ii iii iv
250
98
64
50
36
30

a


kDa
250
98
64
50
36
30

b

Figure 5.8. Graphical representation of the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation in the tail, following incubation in BWW at 37°C for 3 hours with the following treatments: 3 mM PTX; 3 mM PTX + 5mM dbcAMP; 3 mM PTX + 2 mM NADPH (n = 3).



Control

3 mM PTX

3 mM PTX + 5 mM dbcAMP

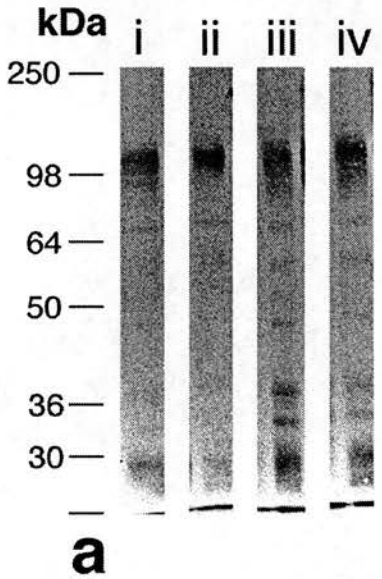
3 mM PTX + 2 mM NADPH

5.3.vii The effect of H_2O_2 and catalase on tyrosine phosphorylation in epididymal rat spermatozoa

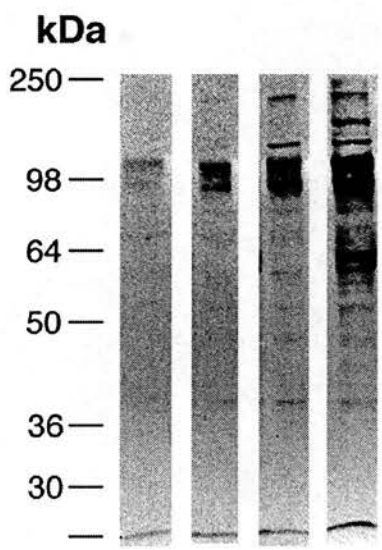
When rat spermatozoa were incubated in the presence of H_2O_2 and catalase, the effects on tyrosine phosphorylation were unexpected. Treatment with H_2O_2 or catalase, either in combination or singularly, appeared to exert little effect on phosphotyrosine expression in caput rat spermatozoa, except for a slight up-regulation in phosphorylation of proteins of approximately 25, 27 and 38 kDa (Fig 5.9a.). In contrast, H_2O_2 induced an up-regulation in tyrosine phosphorylation of proteins within the range of approximately 94-120 kDa, in spermatozoa extracted from the cauda epididymis (Fig 5.9b.). Interestingly, when caudal spermatozoa were incubated with catalase, a scavenger for H_2O_2, an even greater up-regulation in tyrosine phosphorylation was evident (Fig 5.9b.). Proteins within the range of approximately 94-120 kDa were phosphorylated in addition to two novel proteins of approximately 145 and 225 kDa (Fig 5.9b.). Adding a further complexity to these observations was the effect of the combined treatment of H_2O_2 and catalase on the mature caudal sperm cells. Under these conditions proteins of approximately 62, 64, 94-120, 145, 180 and 225 kDa demonstrated increased phosphotyrosine expression (Fig 5.9b.).

These results demonstrate that both H_2O_2 and catalase are capable of inducing tyrosine phosphorylation in mature caudal but not immature caput spermatozoa and this effect was maximised following the combined treatment of these two reagents. However, neither catalase nor H_2O_2 appeared to induce phosphorylation of the tail in either caput or caudal spermatozoa as observed by immunocytochemical analysis (data not shown).

Figure 5.9. Western Blots of tyrosine phosphorylated proteins probed with PY20 following extraction from **(a)** caput and **(b)** caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in BWW at 37°C, for 3 hours with the following treatments: **(i)** control, **(ii)** 100 μM H_2O_2, **(iii)** 5000 U catalase, **(iv)** 100 μM H_2O_2 + 5000 U catalase (n = 3).



a



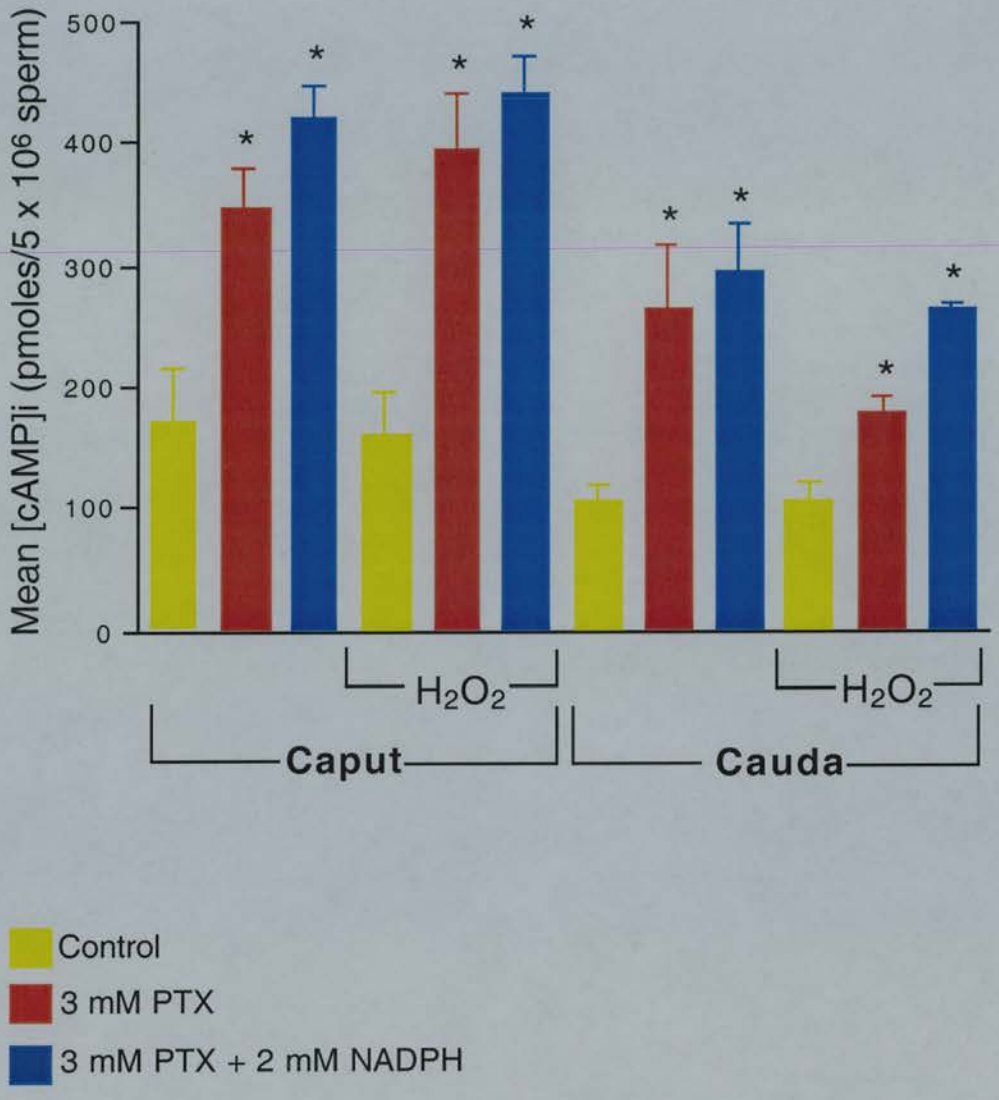
b

5.3.viii The effect of H_2O_2 on intracellular cAMP levels in epididymal rat spermatozoa

To determine whether or not hydrogen peroxide exerted its effect on tyrosine phosphorylation via the cAMP signal transduction pathway, the intracellular concentration of cAMP in both caput and caudal spermatozoa was evaluated as described in Section 5.2.viii.

Hydrogen peroxide was found to exert no effect at all on intracellular cAMP levels in either the immature caput or mature caudal spermatozoa, irrespective of the treatment (Fig. 5.10.). As demonstrated previously, PTX in combination with NADPH significantly increased intracellular cAMP levels in comparison with the controls and spermatozoa incubated with PTX alone (Fig. 5.10.).

Figure 5.10. Data representing the mean intracellular concentration of cAMP in caput and caudal spermatozoa incubated for 3 hours in either nBWW or BWW supplemented with 100 μM H_2O_2, at 37°C with the following treatments: 3 mM PTX; 3 mM PTX + 2 mM NADPH (n = 6).


Mean [cAMP]i (pmoles/5 x 10⁶ sperm)
500
400
300
200
100
0
*
H2O2
H2O2
Caput
Cauda
Control
3 mM PTX
3 mM PTX + 2 mM NADPH

5.4. Discussion

The results presented in this chapter clearly demonstrate that rat epididymal spermatozoa generate ROS both spontaneously and following stimulation with NADPH and NADH, thus emulating observations in other species (Aitken and Clarkson, 1987a; Aitken and Clarkson, 1987b; Fisher and Aitken, 1997; Holland and Storey, 1981; Tosic and Walton, 1946).

This chapter has demonstrated that both caput and caudal epididymal spermatozoa continuously generate relatively equal amounts of $O_2^{\cdot-}$. These data are in agreement with Fisher and Aitken's (Fisher and Aitken, 1997) previously established observations in the rat. They also found that rat spermatozoa generated a steady-state production of $O_2^{\cdot-}$ which did not vary significantly between spermatozoa from different levels of epididymal maturation. In relation to spontaneous $O_2^{\cdot-}$ generation, the rat is very similar to the mouse (Fisher and Aitken, 1997) and although the hamster and guinea pig generated lower levels of $O_2^{\cdot-}$ in comparison, $O_2^{\cdot-}$ production was still independent of epididymal maturation status.

In contrast, when H_2O_2 was measured over a 40 minute period it was evident that caput cells generated insignificant levels in comparison with caudal spermatozoa. This also correlated with what Fisher and Aitken (Fisher and Aitken, 1997) found in the rat, hamster and mouse, as they demonstrated in all three species that the spontaneous generation of H_2O_2 was maximal for spermatozoa released from the cauda epididymis.

The NADPH induced $O_2^{\cdot-}$ response in rat spermatozoa appears to be conserved in the hamster, guinea pig and mouse, as in all of these species NADPH induction of $O_2^{\cdot-}$ was dependent on the epididymal maturation status of the spermatozoa (Fisher and Aitken, 1997). Our current data, together with previous findings (Fisher and Aitken, 1997), suggest that NADPH stimulation of $O_2^{\cdot-}$ is inversely dependent on the maturation status in the rat, in that the response is greater in immature caput spermatozoa in comparison with mature caudal cells and greater still in spermatocytes (Fisher and Aitken, 1997).

These results and previous findings (Aitken and Clarkson, 1987a; Aitken and Clarkson, 1987b; Alvarez and Storey, 1982; Alvarez and Storey, 1984; Alvarez *et al.*, 1987; Bize *et al.*, 1991; Kumar *et al.*, 1990) highlight the universal nature of ROS generation by mammalian spermatozoa suggesting that these molecules have a role to play in the regulation of sperm function. Hydrogen peroxide has been demonstrated to have a positive effect on the process of capacitation (Aitken *et al.*, 1995; Bize *et al.*, 1991; Griveau *et al.*, 1994) and tyrosine phosphorylation (Aitken *et al.*, 1996a; Aitken *et al.*, 1995). Additionally Monteiro and Stern (Monteiro and Stern, 1996) suggested

that peroxide-mediated redox mechanisms were of biological importance in modulating tyrosine phosphorylation-dependent signal transduction pathways in a wide variety of disparate cell types.

The fact that H_2O_2 production is maximal in caudal epididymal spermatozoa suggests that it is important for the mediation of sperm function following the release of these cells from the epididymal environment at ejaculation. Interestingly, H_2O_2 has been demonstrated to reversibly inactivate a low molecular weight phosphotyrosine-protein phosphatase, an enzyme involved in the regulation of some signalling pathways starting from tyrosine kinase receptors (Caselli *et al.*, 1998). Therefore a possible function of H_2O_2 could be to inhibit certain phosphatases resulting in the up-regulation of tyrosine phosphorylation which could be related to the attainment of the capacitated state. The fact that caput spermatozoa generate equal amounts of spontaneous $O_2^{\cdot-}$ and significantly increased amounts of this molecular species in response to NADPH/NADH when compared with caudal cells, suggests that the immature spermatozoa lack the required amount of superoxide dismutase (SOD) necessary for the conversion of $O_2^{\cdot-}$ into H_2O_2. Such a suggestion would be in keeping with the fact that the secreted form of epididymal SOD is only made available to the spermatozoa in the cauda epididymis (Perry *et al.*, 1993).

NADPH has been shown to stimulate tyrosine phosphorylation in human spermatozoa and this was directly correlated with the capacitation status of the spermatozoa (Aitken *et al.*, 1996a; Aitken *et al.*, 1995; Leclerc *et al.*, 1997). As it is evident that both NADPH and NADH stimulate $O_2^{\cdot-}$ in rat spermatozoa it was therefore of interest to establish the effect of these two compounds on tyrosine phosphorylation. In isolation, neither NADPH or NADH appeared to have any effect on tyrosine phosphorylation which was in contrast to observations in human spermatozoa (Aitken *et al.*, 1996a; Aitken *et al.*, 1995). However, when NADPH was combined with PTX tyrosine phosphorylation was up-regulated in caudal spermatozoa. The effect on phosphorylation appeared to be specific to NADPH since neither PTX nor NADH influenced tyrosine phosphorylation in human spermatozoa alone, or in combination, in either the caput or caudal spermatozoa. Also NADPH in combination with PTX increased levels of phosphorylation and [cAMP]i to a greater extent than PTX alone.

As previously demonstrated in Chapter Four, the active cAMP analogue dbcAMP alone does not significantly increase tyrosine phosphorylation. However when caudal spermatozoa are incubated with dbcAMP in combination with PTX, the effect on phosphorylation, localised in the tail is highly significant (refer to Chapter Four). This was also exemplified in the mouse as active cAMP analogues only

significantly up-regulated capacitation-associated protein phosphorylation in the presence of IBMX, a phosphodiesterase inhibitor (Visconti *et al.*, 1995b).

These results suggest that two independent systems are involved in ROS generation by rat spermatozoa; an NADPH oxidase system involved in the redox-regulation of tyrosine phosphorylation and one regulated by NADH. In addition, NADPH seems to exert its effect on tyrosine phosphorylation through the second messenger cAMP as demonstrated by the fact it induces the phosphorylation of proteins localised to the tail that are also phosphorylated by dbcAMP. Further evidence for this hypothesis is provided by the fact that NADPH in combination with PTX significantly raises [cAMP]i in comparison with levels following treatment with PTX alone. Aitken *et al* (Aitken *et al.*, 1998a) previously established that the capacitation-dependent increase in tyrosine phosphorylation in human spermatozoa is controlled by a unique redox-regulated, cAMP-mediated, signal transduction cascade. Although the intracellular systems necessary for redox-regulated tyrosine phosphorylation appear to be active in caput spermatozoa (i.e. they generate high levels of ROS and increased [cAMP]i in response to NADPH), there appears to be an inhibitory mechanism in these cells, preventing cAMP from exerting any effect on the phosphorylation of key proteins. This was also found to be the case when caput spermatozoa were directly stimulated with PTX and dbcAMP as although tyrosine phosphorylation was increased to a certain extent, no induction of phosphorylation of tail proteins was observed.

The effect of zinc chloride, (a tyrosine phosphatase inhibitor, in addition to suppressor of NADPH oxidase activity), on tyrosine phosphorylation was also found to be maturation dependent. Although it induced the phosphorylation of two proteins, of approximately 125 and 225 kDa localised in the tail region of caudal spermatozoa, it either had no effect on caput spermatozoa or even down-regulated phosphorylation of some proteins. A possible explanation is that the active phosphatases in caput spermatozoa are insensitive to zinc chloride inhibition. It is unclear why zinc chloride actually inhibited tyrosine phosphorylation in caput spermatozoa, although zinc chloride is also known to inhibit ROS production (Birnboim and Sandhu, 1997; Carmody *et al.*, 1999; Henderson *et al.*, 1988; Leccia *et al.*, 1993) and this could have a deleterious effect on this process.

Interestingly, zinc chloride only exerted an effect on tyrosine phosphorylation in rat spermatozoa if it was incorporated into the BWW prior to the release of the spermatozoa from the epididymis into the media. When zinc chloride was added to the BWW after the spermatozoa had already been released into the media then no effect at all was observed (data not shown). Evidently spermatozoa become activated

when they are released into BWW and compounds such as zinc chloride are not able to reverse processes that have already begun.

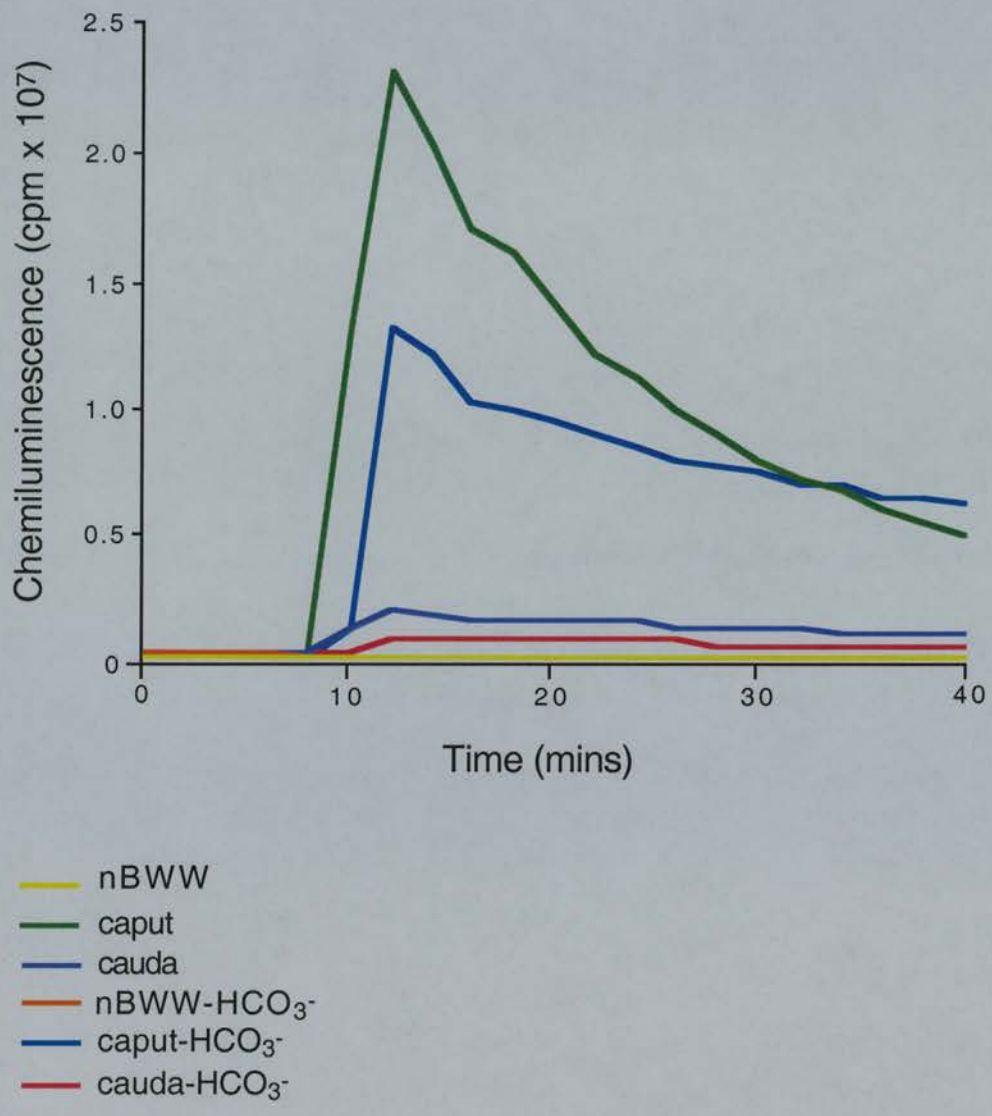
It is difficult to provide an explanation for the observed effects of the direct additions of both H_2O_2 and catalase on tyrosine phosphorylation of rat spermatozoa. Hydrogen peroxide was shown to increase tyrosine phosphorylation, particularly in caudal spermatozoa. However, catalase was also shown to increase tyrosine phosphorylation and the combination of H_2O_2 and catalase was a greater inducer of tyrosine phosphorylation than either reagent alone.

A hypothetical explanation is that added catalase can only scavenge H_2O_2 outwith the cell and O_2 is produced during this process. The oxygen however can move freely into the cell and therefore provide a substrate for the regeneration of ROS within the cell and ultimately H_2O_2, as the cell itself has an intrinsic supply of SOD. Therefore paradoxically the addition of such a scavenger as catalase may actually increase intracellular ROS production leading to an increase in tyrosine phosphorylation.

What remains clear is that however H_2O_2 exerts its action upon tyrosine phosphorylation of rat spermatozoa, it is not mediated by cAMP as no effect on intracellular cAMP levels in rat spermatozoa was observed following incubation with this species. In addition, H_2O_2 did not induce the phosphorylation of proteins located to the tail in rat spermatozoa, irrespective of the region of the epididymis from which they were obtained.

As mentioned earlier in this discussion, H_2O_2 has been demonstrated to reversibly inactivate a low molecular weight phosphotyrosine-protein phosphatase, an enzyme involved in the regulation of some signalling pathways starting from tyrosine kinase receptors (Caselli *et al.*, 1998). This raises the possibility that H_2O_2 may up-regulate tyrosine phosphorylation by the inhibition of certain phosphatases which could be related to the attainment of the capacitated state, while NADPH activates tyrosine phosphorylation through the ability of $O_2^{\cdot-}$ to activate adenylyl cyclase. Interestingly, a paper published by Zhang and Zheng (Zhang and Zheng, 1996) confirmed that $O_2^{\cdot-}$ stimulated intracellular cAMP levels in human spermatozoa.

In conclusion, epididymal maturation of rat spermatozoa is associated with an increased ability to spontaneously generate H_2O_2. In addition, these results indicate that mature rat spermatozoa have developed a unique NADPH oxidase, redox-regulated, cAMP-mediated tyrosine phosphorylation cascade that could play an important role in the attainment of their ability to fertilise an oocyte.


Chemiluminescence (cpm x 10^7)
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
Time (mins)
nBWW
caput
cauda
nBWW-HCO_3^-
caput-HCO_3^-
cauda-HCO_3^-

Chapter Six:

The role of bicarbonate in the *in vitro* support of NADPH-induced ROS generation and tyrosine phosphorylation in rat epididymal spermatozoa

Chapter 6. The role of bicarbonate in the *in vitro* support of NADPH-induced ROS generation and tyrosine phosphorylation in rat epididymal spermatozoa

6.1. Introduction

The tyrosine phosphorylation of proteins in mammalian spermatozoa, has been inextricably associated with the acquisition of motility (Tash, 1989; Tash and Bracho, 1998) and capacitation (Aitken *et al.*, 1998a; Aitken *et al.*, 1995; Visconti *et al.*, 1995a; Visconti *et al.*, 1995b). Capacitation is the poorly defined, post-epididymal maturation process, that is a prerequisite for successful fertilisation (Austin, 1951; Austin, 1952; Chang, 1951; Chang, 1955; Chang, 1984).

In addition to capacitation (Berger and Clegg, 1983; Monks *et al.*, 1986; Stein and Fraser, 1984), changes in protein phosphorylation have been implicated in the induction of the acrosome reaction (Garbers and Kopf, 1980; Kopf and Gerton, 1991).

The role of cAMP in the regulation of tyrosine phosphorylation is well documented in various species (Aitken *et al.*, 1998a; Bookbinder *et al.*, 1991; Brandt and Hoskins, 1980; Kalab *et al.*, 1998; Leclerc *et al.*, 1996; MacLeod *et al.*, 1994; Mahoney and Gwathmey, 1999; Tash and Means, 1983; Vijayaraghavan *et al.*, 1997a; Visconti *et al.*, 1995a; Visconti and Kopf, 1998; Visconti *et al.*, 1995b). Moreover, the results documented in this thesis (refer to Chapter 4) have clearly demonstrated that this process is also regulated by cAMP in the rat.

One of the best known intracellular activators of sperm adenylyl cyclase is believed to be bicarbonate (Okamura *et al.*, 1991; Okamura *et al.*, 1985). This is supported further by the inhibitory effect of $NaHCO_3^-$-free media on tyrosine phosphorylation and capacitation in the mouse (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b). $NaHCO_3^-$-free conditions were also found to inhibit ROS generation, tyrosine phosphorylation and the capacity to generate normal calcium transients in response to progesterone in human spermatozoa (Aitken *et al.*, 1998b). In hamster sperm, $NaHCO_3^-$-free incubation conditions were found to inhibit the phorbol 12-myristate 13-acetate (PMA) stimulated elevation of [cAMP]i (Visconti *et al.*, 1990). As well as stimulating [cAMP]i accumulation (Visconti *et al.*, 1990), phorbol esters such as PMA are also known to induce a $O_2^{\cdot-}$ burst in human spermatozoa (Aitken and Buckingham,

1992b; Aitken *et al.*, 1992a). Additionally, HCO_3^- has been found to necessary for the initiation and maintenance of hyperactivated motility in many species of mammalian sperm (Si, 1996; Si, 1997). It is thought to stimulate hyperactivation of spermatozoa, by elevating [cAMP]i through the direct stimulation of adenylate cyclase (Si, 1996; Si, 1997). HCO_3^- has also been implicated in the progesterone-induced human sperm acrosome reaction (Sabeur and Meizel, 1995).

The primary aim of this chapter was to evaluate the relationship between redox-cycling and cAMP-mediated tyrosine phosphorylation in rat spermatozoa and also to determine the importance of $NaHCO_3^-$ on these two processes *in vitro*. A secondary aim was to evaluate the effects of removing the additional media constituents of albumin and glucose, on tyrosine phosphorylation in rat epididymal spermatozoa. It is broadly accepted that capacitation can be achieved *in vitro* in balanced salt solutions containing appropriate concentrations of electrolytes, metabolic energy sources and serum albumin (Yanagimachi, 1994). The requirement for an energy source is self-explanatory in that it is crucial for the maintenance of motility and the metabolism of the sperm cell. It has been postulated that albumin is responsible for the removal of cholesterol from the sperm plasma membrane (Davis, 1976; Davis, 1980b; Davis *et al.*, 1980a; Davis *et al.*, 1979; Go and Wolf, 1985; Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989). One possibility is that this could be related to the membrane fluidity changes that have been documented in many species of sperm during capacitation (Yanagimachi, 1994). In addition, serum albumin has been reported to be necessary for hyperactivation of sperm motility in various species of mammalian sperm (Si, 1996; Si, 1997). Consequently it was of interest to establish if those media conditions that have been shown to support capacitation are also required for the *in vitro* support of tyrosine phosphorylation in the rat.

6.2. Materials and Methods

Refer to Chapter 2 for general materials and methods.

6.2.i. The effect of bicarbonate free BWW (HCO_3^--free BWW) on NADPH-induced superoxide ($O_2^{\cdot-}$) generation in rat epididymal spermatozoa

As detailed in Section 2.6., detection of $O_2^{\cdot-}$ was measured by lucigenin-dependent chemiluminescence using Berthold luminometers (LB9505, Berthold Analytical Instruments, Wildbad, Germany) at 37°C. Lucigenin is a charged compound used for the detection of superoxide. It is relatively membrane impermeant and thus largely measures the release of production of $O_2^{\cdot-}$ to the extracellular space.

HCO_3^--free BWW was made up as normal except that the 25 mM $NaHCO_3^-$ was replaced with 25 mM NaCl in order to maintain the osmolarity of the medium. Rat spermatozoa were extracted from the epididymis as described in Section 2.3., except that sperm from one epididymis were released into normal BWW and those from the remaining organ released into HCO_3^--free BWW. Motility and density counts (Sections 2.4. and 2.5. respectively) were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The procedure for detection of spontaneous $O_2^{\cdot-}$ generation by rat epididymal spermatozoa was carried out exactly as described in Section 2.6.i. When the addition of NADPH was required, the luminometer run was temporarily halted and NADPH diluted in BWW was added to the appropriate cuvettes, at a final concentration of 2 mM. Immediately following the addition of NADPH, the luminometer run was restarted. Both normal BWW and HCO_3^--free BWW without sperm were used as controls in each luminometer run.

6.2.ii The effect of HCO_3^--free BWW on tyrosine phosphorylation in epididymal rat spermatozoa

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one of the caput epididymis was diluted into normal BWW while those from the other were diluted straight into HCO_3^--free BWW. The same procedure was then carried out on the spermatozoa from the caudal region. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.). The spermatozoa were incubated with

various treatments including 3 mM PTX, 2 mM NADPH and 5 mM dbcAMP for 3 hours at 37°C. On completion of the three hour incubation period, rat sperm proteins were extracted using SDS as described in Section 2.7. and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11.

6.2.iii The effect of HCO_3^--free BWW on intracellular cAMP levels in epididymal rat spermatozoa

Following the release of rat spermatozoa into both normal BWW and HCO_3^--free BWW as described above in Sections 6.2.i. and 6.2.ii, motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. Spermatozoa were incubated at this concentration with either 3 mM PTX or the combined treatment of 3 mM PTX and 2 mM NADPH for 3 hours at 37°C. Following completion of the incubation period, extraction of cAMP from the spermatozoa was performed as described in Section 2.15.

6.2.iv The influence of pH on the HCO_3^- mediated effects exerted on tyrosine phosphorylation of rat epididymal spermatozoa

HCO_3^--free BWW (pH 8.4) was made up the same as HCO_3^--free BWW, except that 20 mM Hepes buffer (Gibco, UK) was replaced with 20 mM N-Tris[Hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS) and the media pH was adjusted to 8.4 with concentrated NaOH. The pH of normal BWW was 7.6.

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one epididymis were diluted in HCO_3^--free BWW at a pH of 7.6. while those from the other were released into HCO_3^--free BWW with TAPS at a pH of 8.4. Motilities and densities were carried out and the cells adjusted to a concentration of 10 x 10^6/ml (Sections 2.4. and 2.5.). The spermatozoa were incubated with and without 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C.

On completion of the incubation period, the sperm were processed for Western Blot analysis as described in Sections 2.7.-2.11.

The above experiment was repeated using normal complete BWW (pH 7.6.) and complete BWW (pH 8.4.) as alternatives to the above mentioned HCO_3^--free media.

6.2.v The effect of glucose-free BWW on tyrosine phosphorylation of rat epididymal spermatozoa

Glucose-free BWW was made up as described in Chapter 2, except that glucose was replaced by the inactive analogue, 2-deoxyglucose at the same concentration of 5.6 mM.

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one epididymis were diluted in normal BWW while those from the other were released into glucose-free BWW. Motilities and densities were carried out and the cells adjusted to a concentration of 10 x 10^6/ml (Sections 2.4. and 2.5.). The spermatozoa were incubated with and without 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C.

On completion of the incubation period, the sperm were processed for Western Blot analysis as described in Sections 2.7.-2.11.

6.2.vi The effect of albumin-free BWW on tyrosine phosphorylation of rat epididymal spermatozoa

Albumin-free BWW consisted of the usual constituents of BWW except that albumin was replaced with polyvinylalcohol (PVA) at a concentration of 1 mg/ml.

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one epididymis were diluted in normal BWW while those from the other were released into albumin-free BWW. Motilities and densities were carried out and the cells adjusted to a concentration of 10 x 10^6/ml (Sections 2.4. and 2.5.). The spermatozoa were incubated with and without 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C.

On completion of the incubation period, the sperm were processed for Western Blot analysis as described in Sections 2.7.-2.11.

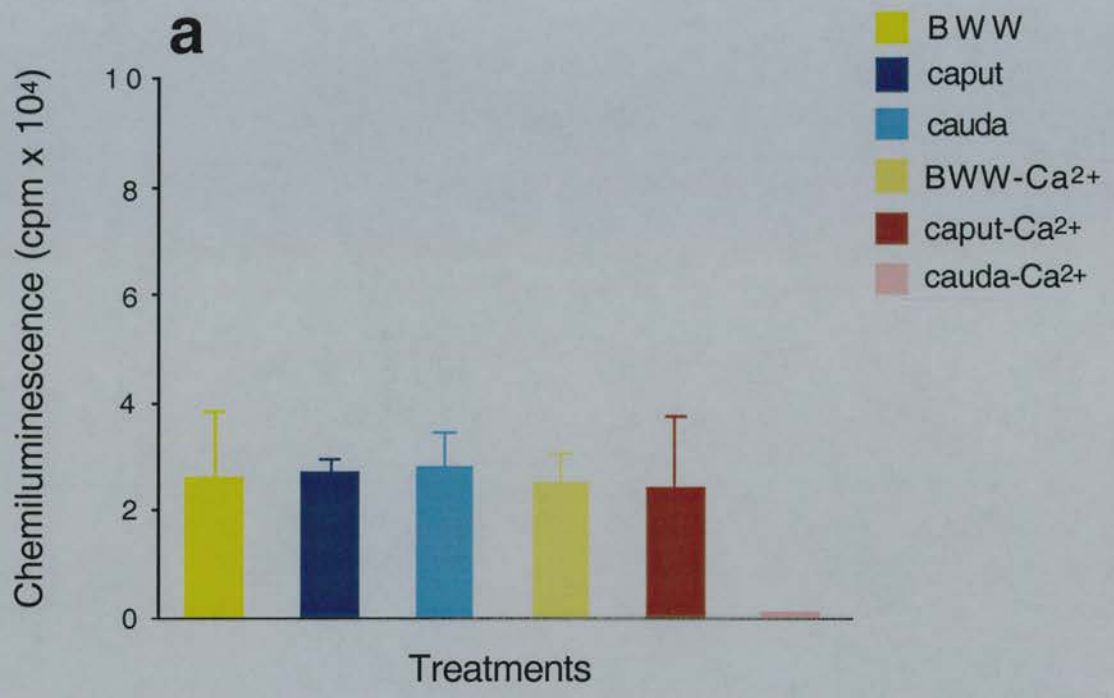
6.3. Results

6.3.i. The effect of bicarbonate free BWW (HCO_3^--free BWW) on NADPH-induced superoxide ($O_2^{-\cdot}$) generation in rat epididymal spermatozoa

As our current results together with previous findings (Aitken *et al.*, 1995) suggest that tyrosine phosphorylation may be a redox-regulated process we thought it would be of interest to investigate the effect of HCO_3^--free BWW on NADPH induced $O_2^{-\cdot}$ generation and tyrosine phosphorylation of rat epididymal spermatozoa. Epididymides from one animal were used in the same experiment and spermatozoa from one organ were released into complete BWW and spermatozoa from the other organ were released into HCO_3^--free BWW.

There was no significant difference in spontaneous $O_2^{-\cdot}$ generation between any of the spermatozoa whether suspended in normal BWW or HCO_3^--free BWW (Fig. 6.1. and 6.2a.). When the cells were stimulated with 2 mM NADPH it was evident that HCO_3^--free BWW exerted a negative effect on the NADPH induced $O_2^{-\cdot}$ response. Both the caput and caudal spermatozoa in complete BWW generated a greater amount of $O_2^{-\cdot}$ in response to NADPH than their corresponding samples in HCO_3^--free BWW, although the difference was only statistically significant in the caput spermatozoa (Fig. 6.1. and 6.2.). On completion of the 40 minute luminometer run, levels of $O_2^{-\cdot}$ had equalled out as although they were still reduced in the sperm incubated in HCO_3^--free BWW, the difference was no longer significant between either the caput or caudal spermatozoa (Fig. 6.2e.).

Figure 6.1. The effect of HCO_3^--free BWW on 2 mM NADPH-induced lucigenin-dependent chemiluminescent $O_2^{\cdot-}$ generation in both caput and caudal spermatozoa. 2 mM NADPH was added to both complete BWW and $NaHCO_3^-$-free BWW for use as controls (n = 3).


a
BWW
caput
cauda
BWW-Ca2+
caput-Ca2+
cauda-Ca2+
Chemiluminescence (cpm x 10⁴)
10
8
6
4
2
0
Treatments

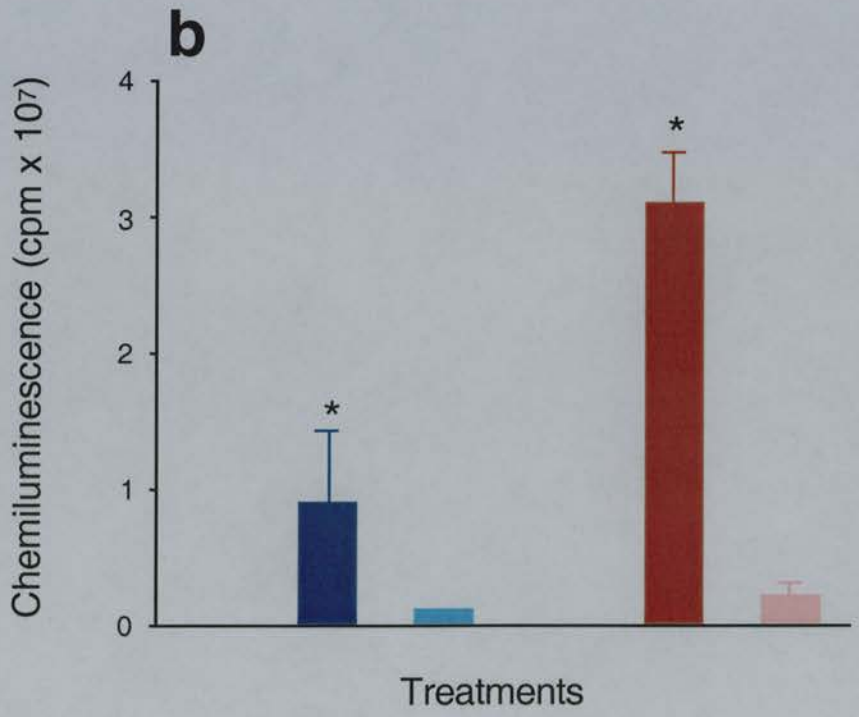
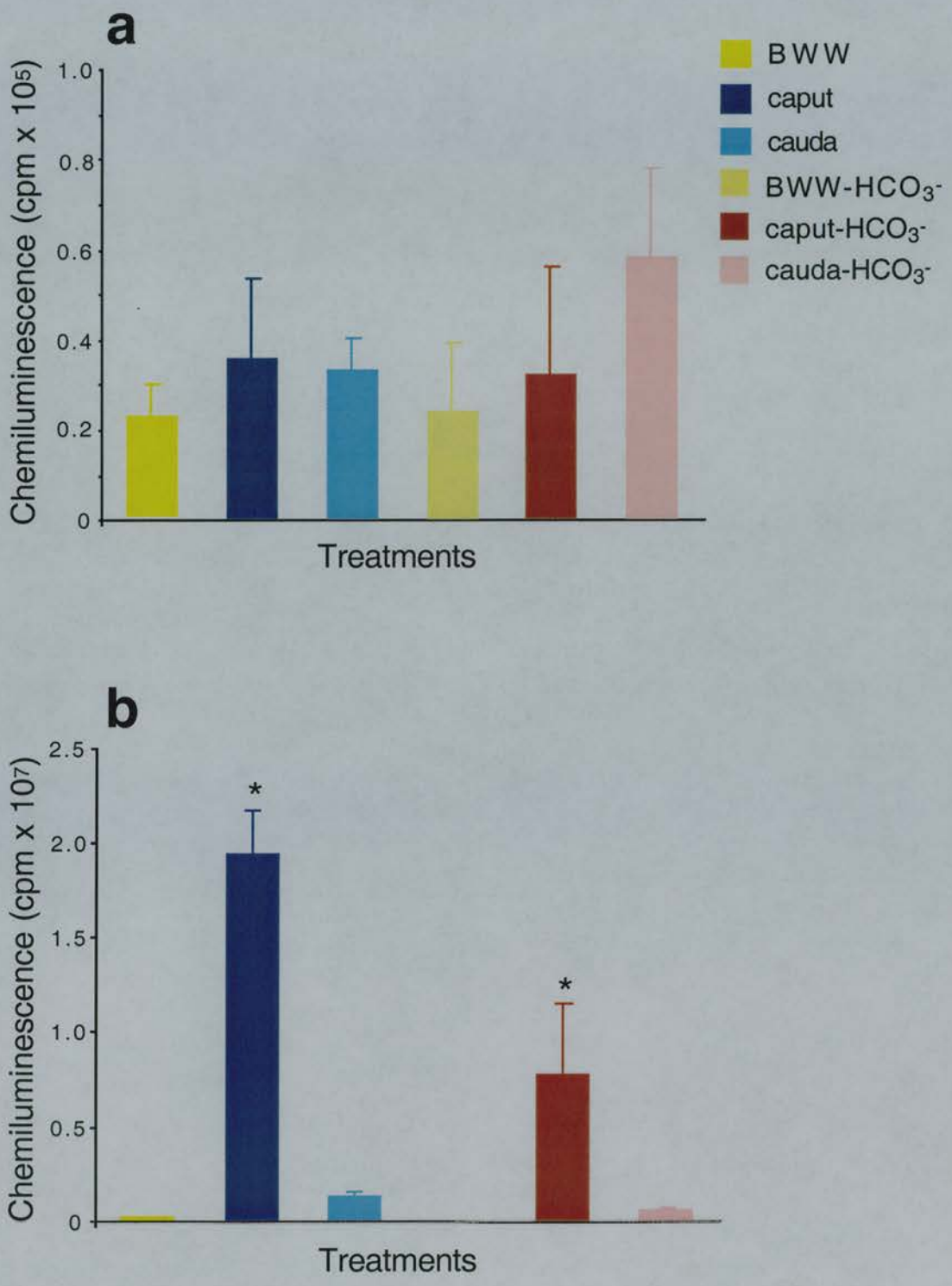
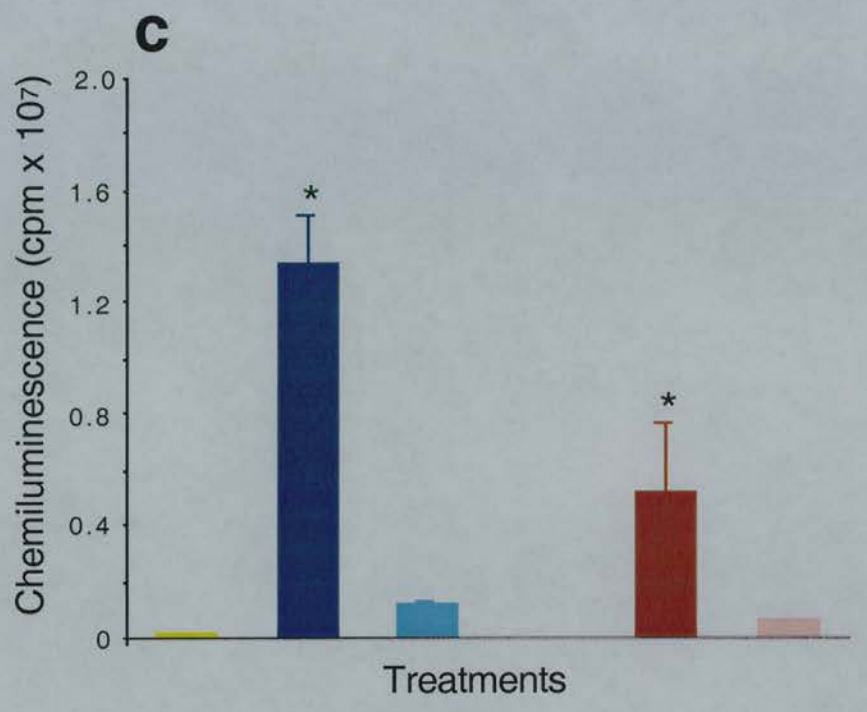
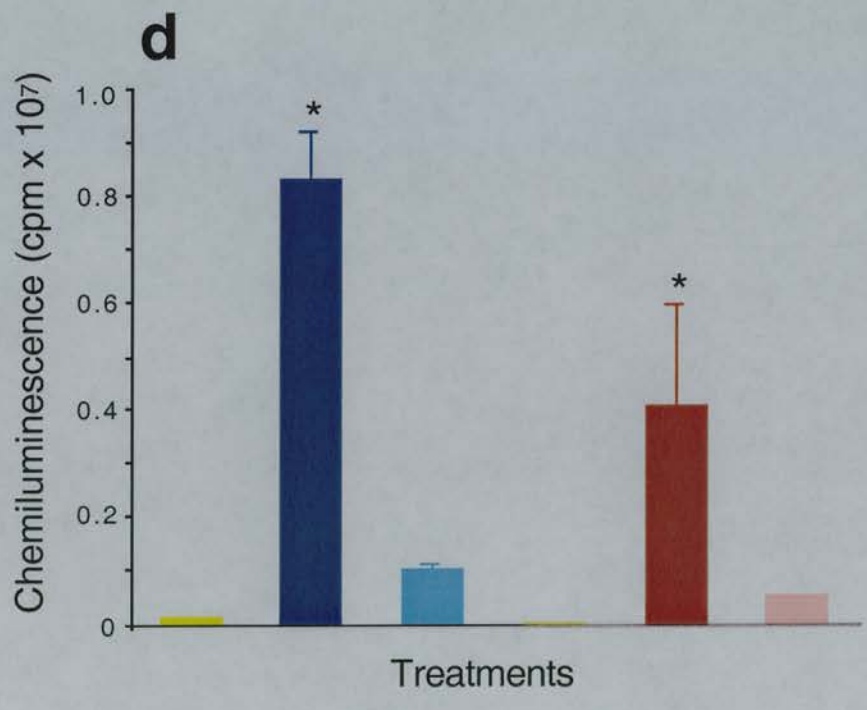
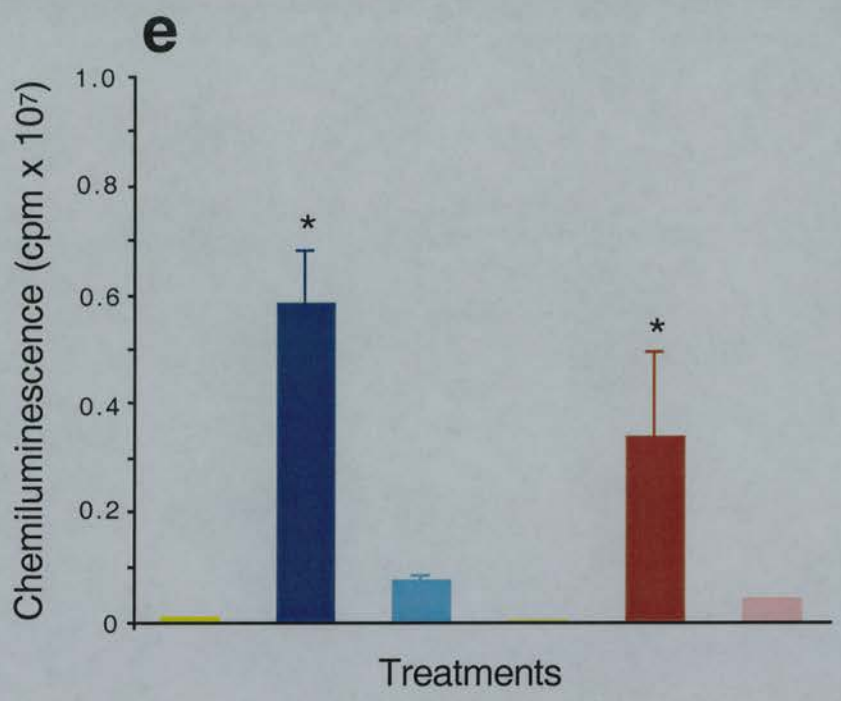

b
Chemiluminescence (cpm x 10⁷)
4
3
2
1
0
*
*
Treatments

Figure 6.2. The following bar charts represent the mean production of $O_2^{-\cdot}$ in response to NADPH in rat spermatozoa, incubated in both complete BWW and HCO_3^- -free BWW. Superoxide induced chemiluminescence was measured in counts per minute (cpm) following **(a)** 0 minutes, **(b)** 12 minutes, **(c)** 20 minutes, **(d)** 30 minutes and **(e)** 40 minutes incubation (n = 3).


a
Chemiluminescence (cpm x 10⁵)
1.0
0.8
0.6
0.4
0.2
0
Treatments
BWW
caput
cauda
BWW-HCO3-
caput-HCO3-
cauda-HCO3-
b
Chemiluminescence (cpm x 10⁷)
2.5
2.0
1.5
1.0
0.5
0
*
*
Treatments


c
Chemiluminescence (cpm x 10⁷)
2.0
1.6
1.2
0.8
0.4
0
*
*
Treatments


d
Chemiluminescence (cpm x 10⁷)
1.0
0.8
0.6
0.4
0.2
0
*
*
Treatments


e
Chemiluminescence (cpm x 10⁷)
1.0
0.8
0.6
0.4
0.2
0
*
*
Treatments

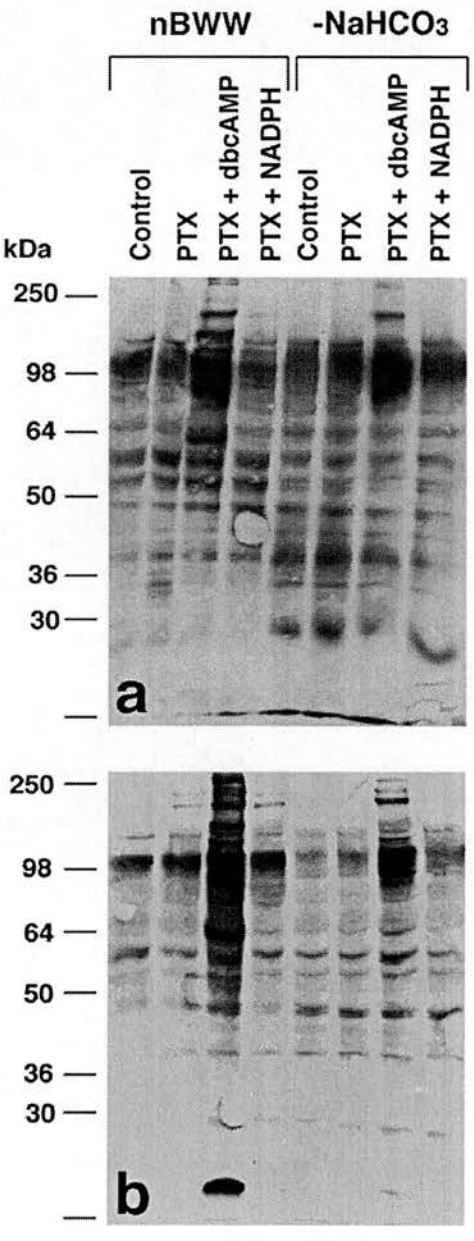
6.3.ii The effect of HCO_3^--free BWW on tyrosine phosphorylation in epididymal rat spermatozoa

The effect of HCO_3^--free BWW on tyrosine phosphorylation was interesting, as once again the pattern of tyrosine phosphorylated proteins differed between caput and caudal spermatozoa.

Spermatozoa were incubated in various treatments for 3 hours at 37°C in the two types of BWW. Sperm proteins were then extracted with 1% SDS as described in Chapter 2. The effect of HCO_3^--free BWW on tyrosine phosphorylation in caput spermatozoa was not as great as observed in the caudal cells (Fig. 6.3.). There was a reduction in tyrosine phosphorylation of the high molecular weight proteins (above approximately 132 kDa) and also those ranging from approximately 50-64 kDa (Fig. 6.3a.). However, there was actually a slight increase in phosphorylation of some of the lower molecular weight proteins within the range of approximately 26-48 kDa (Fig. 6.3a.).

The effect of HCO_3^--free BWW on tyrosine phosphorylation in caudal spermatozoa was similar to what Visconti *et al.*, 1995a, observed in the mouse whereby tyrosine phosphorylation was virtually eliminated apart from the p95/116 hexokinase, which was independent of HCO_3^- concentration. This was also the case in rat caudal spermatozoa as there was a reduction in tyrosine phosphorylation of most proteins apart from three proteins of approximately 45, 52 and 55 kDa (Fig. 6.3b.). The addition of 3 mM PTX and 5 mM dbcAMP overcame the negative effect of HCO_3^--free BWW on tyrosine phosphorylation to a certain extent, but the intensity of phosphorylation was still greatly reduced in comparison with the same treatment in complete BWW (Fig. 6.3b.). This was also similar to what Visconti *et al.*, 1995b observed in the mouse, except that they found that the combination of a phosphodiesterase inhibitor and cAMP analogue completely overcame the effect of a lack of HCO_3^-.

Figure 6.3. Western Blots of tyrosine phosphorylated proteins probed with PY20 following extraction from **(a)** caput spermatozoa and **(b)** caudal spermatozoa using 1% SDS. The spermatozoa had previously been incubated in either complete BWW or $NaHCO_3^-$-free BWW, at 37°C for 3 hours with the following treatments: 3 mM PTX; 3 mM PTX + 2 mM NADPH; 3 mM PTX + 5mM dbcAMP (n = 5).

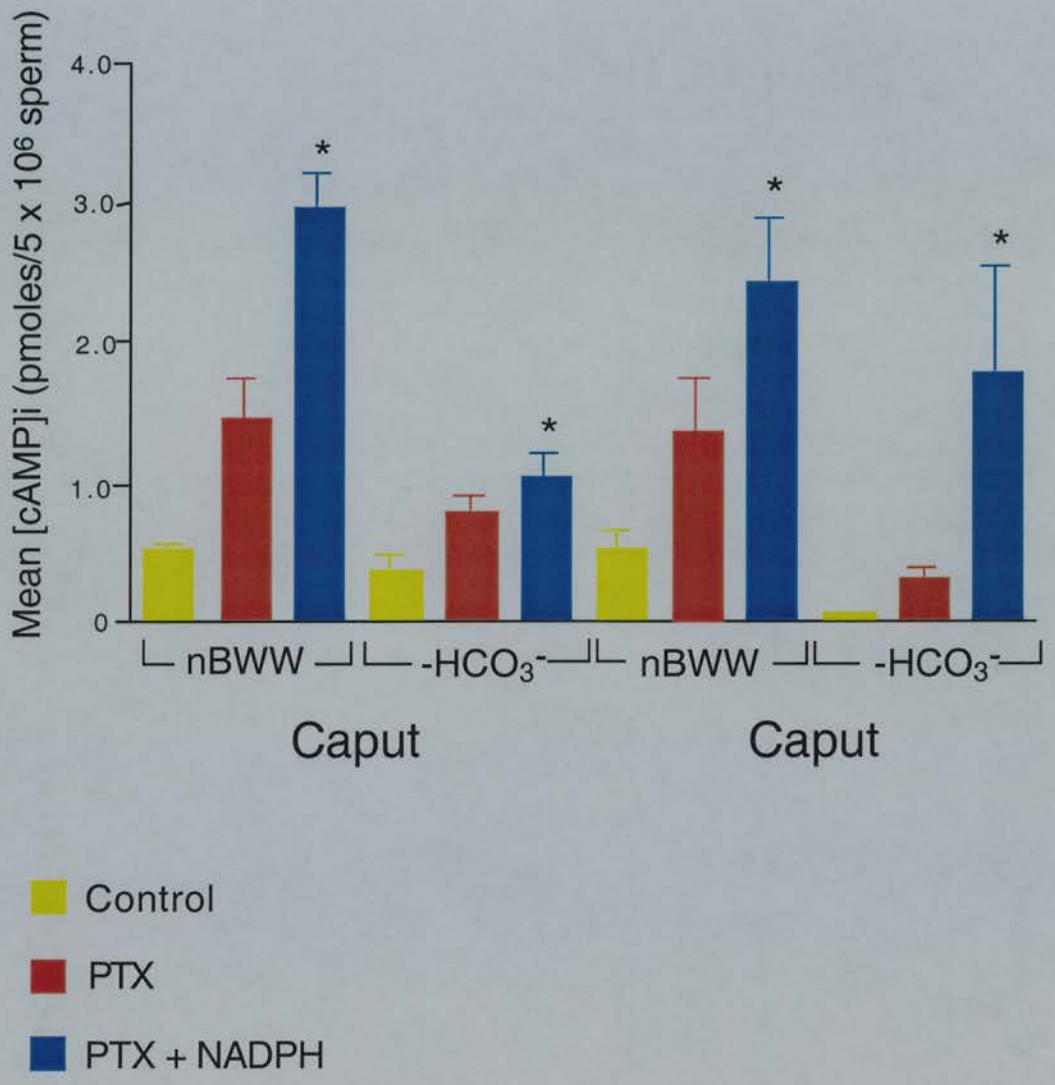

nBWW
-NaHCO3
Control
PTX
PTX + dbcAMP
PTX + NADPH
Control
PTX
PTX + dbcAMP
PTX + NADPH
kDa
250
98
64
50
36
30
a
250
98
64
50
36
30
b

6.3.iii The effect of HCO_3^--free BWW on intracellular cAMP levels in epididymal rat spermatozoa

When intracellular cAMP was evaluated in rat spermatozoa that had been incubated in HCO_3^--free BWW, it was evident that HCO_3^- does indeed exert an effect on [cAMP]i (Fig. 6.4.).

All of the spermatozoa incubated with both 3 mM PTX and 2 mM NADPH had a significantly higher [cAMP]i than those treated with 3 mM PTX only or the controls. Only caput spermatozoa treated with both PTX and NADPH in HCO_3^--free BWW did not have a statistically significant higher [cAMP]i than those treated with 3 mM PTX alone (Fig. 6.4.). All of the treated immature caput spermatozoa suspended in HCO_3^--free BWW demonstrated reduced [cAMP]i, in comparison with those treated in complete BWW. However, this was only statistically significant in the PTX and NADPH treated spermatozoa. Similarly, the caudal cells in HCO_3^--free BWW also demonstrated reduced [cAMP]i in comparison with those treated in complete BWW, although this was only statistically significant in caudal cells treated with PTX alone.

Figure 6.4. Data representing the mean intracellular concentration of cAMP in caput and caudal spermatozoa incubated in either BWW or HCO_3^--free BWW for 3 hours at 37°C with the following treatments: 3 mM PTX; 3 mM PTX + 2 mM NADPH (n = 6).


Mean [cAMP]i (pmoles/5 x 10⁶ sperm)
4.0
3.0
2.0
1.0
0
*
*
*
*
nBWW
-HCO3-
nBWW
-HCO3-
Caput
Caput
Control
PTX
PTX + NADPH

6.3.iv The influence of pH on the HCO_3^- mediated effects exerted on tyrosine phosphorylation of rat epididymal spermatozoa

In addition to the obvious deleterious effects of lack of bicarbonate on $O_2^{\cdot-}$ generation, tyrosine phosphorylation and [cAMP]i it was also evident that the effect on phosphorylation could be due, partly, to a reduction in extracellular pH. When rat spermatozoa were incubated in HCO_3^--free BWW that had been buffered to a pH of 8.4 with TAPS, tyrosine phosphorylation was significantly up-regulated in comparison with spermatozoa that had been incubated in HCO_3^--free BWW at the normal pH of 7.6 (Fig. 6.5.). This suggests that an increase in the pH of the extracellular media can override the negative effect of HCO_3^--free BWW on tyrosine phosphorylation.

In addition when rat spermatozoa were incubated in complete BWW buffered to a pH of 8.4., the results clearly demonstrated that by raising the extracellular pH it is possible to induce the phosphorylation of tyrosine residues on a large number of different proteins in both caput and caudal spermatozoa (Fig. 6.6.). This is in direct correlation with what was demonstrated in spermatozoa that had been incubated in HCO_3^--free BWW that had been buffered to a pH of 8.4 with TAPS (Fig. 6.5.). The data confirms further, that increased pH has a beneficial effect on tyrosine phosphorylation and can override the suppressive effects due HCO_3^- removal.

Figure 6.5. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either HCO_3^--free BWW buffered to pH 8.4 (A) or, $NaHCO_3^-$-free BWW (B), at 37°C for 3 hours with 3 mM PTX + 5mM dbcAMP (n = 4).

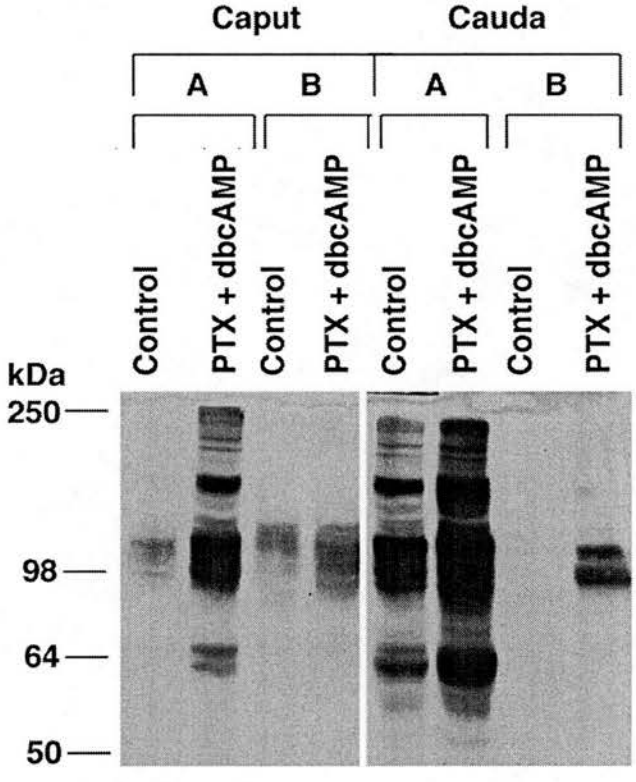
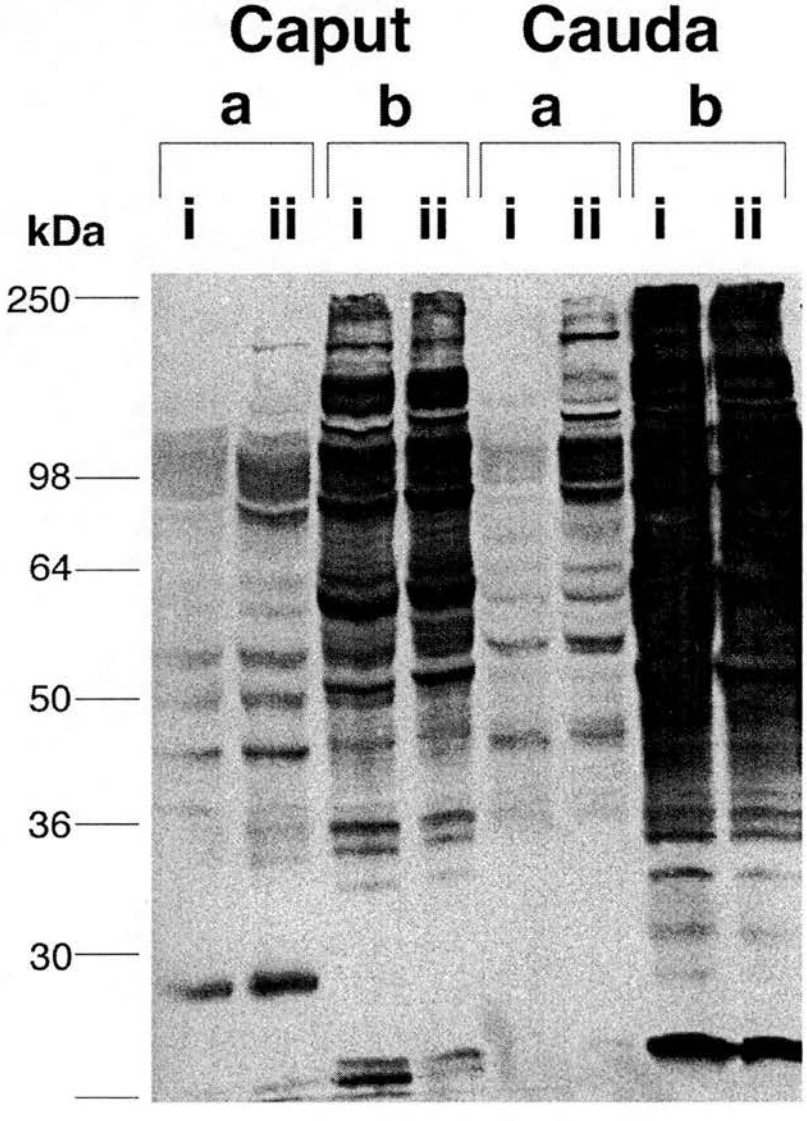

Caput
Cauda
A
B
A
B
Control
PTX + dbcAMP
Control
PTX + dbcAMP
Control
PTX + dbcAMP
Control
PTX + dbcAMP
kDa
250
98
64
50

Figure 6.6. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either **(a)** normal BWW buffered to a pH of 7.6 with Hepes or, **(b)** normal BWW buffered to pH 8.4 with TAPS, at 37°C for 3 hours. In addition the cells were incubated with the following treatments: **(i)** control and **(ii)** 3 mM PTX + 5mM dbcAMP (n = 3).


Caput
Cauda
a
b
a
b
kDa
i
ii
i
ii
i
ii
i
ii
250
98
64
50
36
30

6.3.v The effect of glucose-free BWW on tyrosine phosphorylation of rat epididymal spermatozoa

As expected, when the energy source glucose was exchanged for the inactive analogue 2-deoxyglucose in the incubation media, down-regulation of tyrosine phosphorylation of virtually all proteins was evident (Fig. 6.7.). Even the addition of the stimulators PTX and dbcAMP could not override the inhibitory effects of 2-deoxyglucose on this process in rat epididymal spermatozoa (Fig. 6.7.).

6.3.vi The effect of albumin-free BWW on tyrosine phosphorylation of rat epididymal spermatozoa

Reports on mouse spermatozoa demonstrated that an absence of BSA in capacitation media lead to the inhibition of tyrosine phosphorylation in all proteins except for the p95/116 hexokinase (Visconti *et al.*, 1995a). The results presented here in the rat however, suggest that the absence of albumin exerted a limited effect on this process in comparison.

In caput spermatozoa, there was actually a slight increase in the intensity of phosphorylation of proteins of approximately 98-130 kDa, following incubation in albumin-free BWW (Fig. 6.8.). In addition phosphorylation of two novel proteins of approximately 18 and 86 kDa was evident irrespective of the treatment, under these albumin-free conditions (Fig. 6.8.). However, in the PTX and dbcAMP treated caput cells tyrosine phosphorylation of a protein of approximately 225 kDa was inhibited (Fig. 6.8.).

Under these albumin-free conditions, untreated caudal spermatozoa demonstrated an increase in tyrosine phosphorylation of proteins of approximately 57, 86 and 98 kDa (Fig. 6.8.). However, when the treated caudal spermatozoa incubated in the two different media were compared, it was evident that the albumin-free BWW lead to the down-regulation of phosphorylation of proteins within the approximate ranges of 46-60 and 68-82 kDa.

Figure 6.7. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either **(a)** normal BWW or, **(b)** glucose-free BWW with 2-deoxyglucose, at 37°C for 3 hours. In addition the cells were incubated with the following treatments: **(i)** control and **(ii)** 3 mM PTX + 5mM dbcAMP (n = 3).

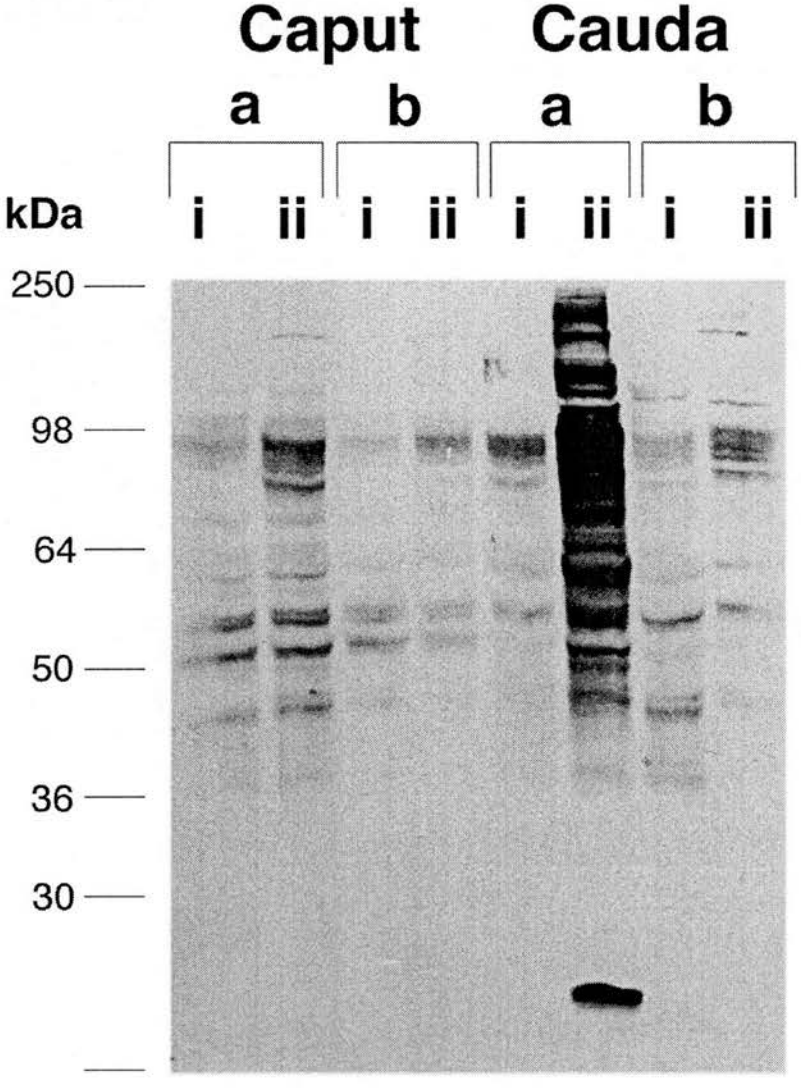
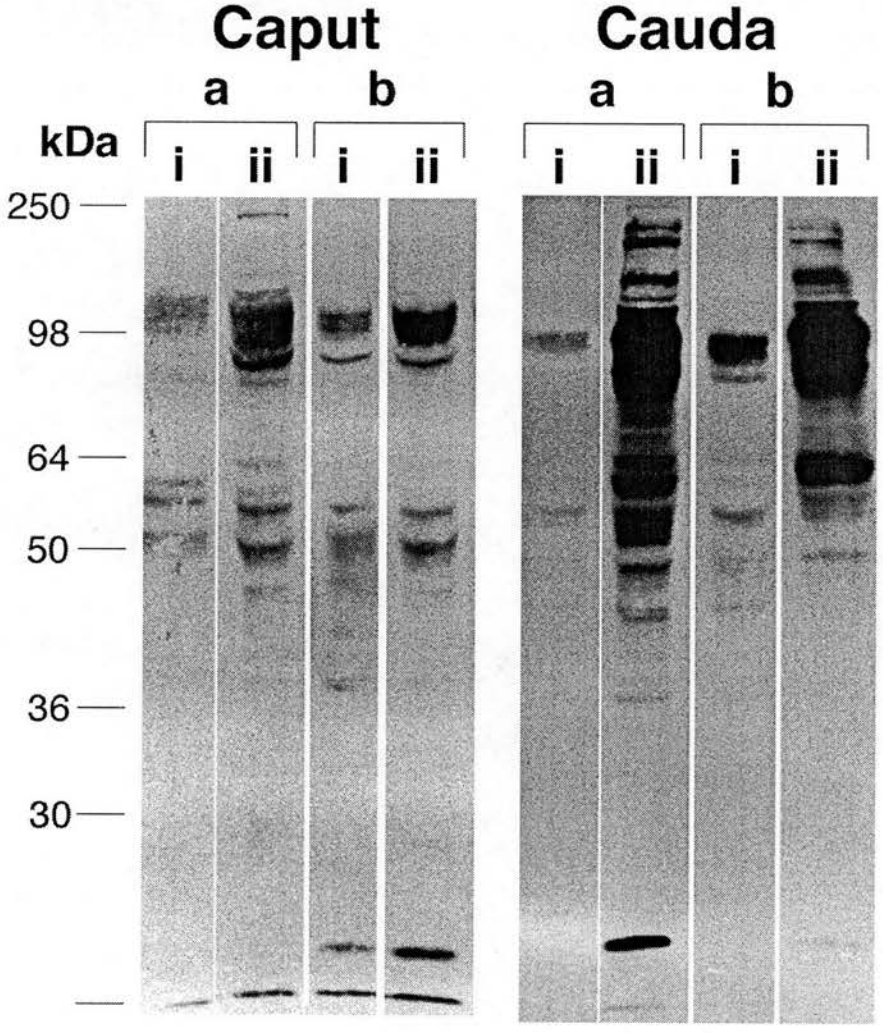

Caput
Cauda
a
b
a
b
kDa
i
ii
i
ii
i
ii
i
ii
250
98
64
50
36
30

Figure 6.8. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either **(a)** normal BWW or, **(b)** albumin-free BWW with PVA, at 37°C for 3 hours. In addition the cells were incubated with the following treatments: **(i)** control and **(ii)** 3 mM PTX + 5mM dbcAMP (n = 3).


Caput
Cauda
a
b
a
b
kDa
i
ii
i
ii
i
ii
i
ii
250
98
64
50
36
30

6.4. Discussion

Various laboratories have demonstrated the importance of the inclusion of HCO_3^- in capacitating media (Aitken *et al.*, 1998b; Boatman and Robbins, 1991; Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Visconti and Kopf, 1998; Visconti *et al.*, 1990). The requirement of HCO_3^- in the incubation media for the capacitation associated changes in tyrosine phosphorylation has also been demonstrated in the mouse (Visconti *et al.*, 1995a) and human (Aitken *et al.*, 1998b).

In this present study the effect of HCO_3^--free BWW on NADPH-induced $O_2^{\cdot-}$ generation, tyrosine phosphorylation and [cAMP]i was evaluated in rat epididymal spermatozoa. The results clearly demonstrated that the removal of HCO_3^- significantly reduced NADPH-induced $O_2^{\cdot-}$ generation in both caput and caudal spermatozoa as well as tyrosine phosphorylation and [cAMP]i.

The reduced ability of NADPH to stimulate an increase in intracellular cAMP in HCO_3^--free BWW could be causally related to the reduced capacity of this coenzyme to stimulate ROS generation. The latter has been shown to stimulate cAMP generation in human (Aitken *et al.*, 1998a; Zhang and Zheng, 1996) and rat spermatozoa (this study), presumably through a direct effect on adenylyl cyclase activity, given the additive effects of NADPH and PTX on [cAMP]i and tyrosine phosphorylation. Alternatively, the loss of $O_2^{\cdot-}$ generating capacity and the impaired cAMP response observed in HCO_3^--free BWW may be independent reflections of the importance of intracellular pH in the control of sperm biochemistry and the central role that HCO_3 plays in this context.

The fact that all aspects of sperm function could be restored to normal if the HCO_3^--free medium was buffered to pH 8.4, strongly supports the notion that the impact of this anion on sperm function is mediated largely, if not exclusively, by its impact on cytosolic pH. This conflicts with observations in the mouse whereby the combined action of IBMX and an active cAMP analogue could completely override the deleterious effect of HCO_3^--free conditions on tyrosine phosphorylation (Visconti *et al.*, 1995b). Such data support the notion that, in the mouse at least, HCO_3^- is required to maintain cAMP generation. However, in the rat, even if a maximal cAMP stimulus (3 mM PTX and 5 mM dbcAMP) was applied to spermatozoa incubated in HCO_3^--free medium, tyrosine phosphorylation was still suppressed.

In similar fashion to the rat, the impact of HCO_3^- on human sperm function appears to be entirely mediated by changes in intracellular pH (Aitken et al., 1998). Thus in a recent study HCO_3^--free conditions reduced ROS generation by human

spermatozoa, tyrosine phosphorylation status, the capacity of these cells to generate normal calcium transients on exposure to progesterone and ultimately their ability to fuse with the oocyte (Aitken *et al.*, 1998b). When the pH of the medium was raised to pH 8.4 in order to restore intracellular pH to its normal value of around 7.3. all aspects of sperm function, including tyrosine phosphorylation status, progesterone induced calcium transients and potential for oocyte fusion, were normalised despite the absence of HCO_3^- in the extracellular medium (Aitken *et al.*, 1998b).

The results obtained in the present study appear to mirror those obtained in the human in suggesting that extracellular HCO_3^- mediates its effects on rat sperm biochemistry through its impact on intracellular pH. Not only is the capacity for ROS generation in these cells sensitive to changes in intracellular pH, but the PKA-activated tyrosine kinase may also be sensitive to H^+ concentration, given the inability of dbcAMP to fully restore tyrosine phosphorylation in HCO_3^- free medium.

It should be emphasised that even in HCO_3^--free medium, the spermatozoa are not completely free of this anion. Sperm possess carbonic anhydrase activity (Parkkila *et al.*, 1991) and in the presence of this enzyme, carbon dioxide generated as a consequence of respiration will be converted into HCO_3^-. This intracellularly generated HCO_3^- is presumably sufficient to provide rat sperm adenylyl cyclase with sufficient quantities of this anion to remain active and, in the case of caudal spermatozoa, promote vigorous motility. However every molecule of HCO_3^- generated in this way will be accompanied by a proton ($H_2O + CO_2 = H_2CO_3 = H^+ + HCO_3^-$) and intracellular pH will remain low. In contrast every molecule of extracellular HCO_3^- that enters the cell from the extracellular space has the potential to consume a proton and, as a consequence, intracellular pH will rise.

In addition to bicarbonate, the presence of an energy source and albumin in the incubation media are thought to be crucial for the acquisition of the capacitated state in spermatozoa under *in vitro* conditions (Yanagimachi, 1994). It was therefore of interest to investigate as part of this study, the effects of the removal of these media constituents on tyrosine phosphorylation in rat epididymal spermatozoa.

Not unexpectedly, the replacement of glucose with 2-deoxyglucose in the incubation media exerted a highly inhibitory effect on the process of protein phosphorylation. Due to the compact nature and reduced cytoplasmic space inherent to spermatozoa, these highly specialised cells have limited energy resources and consequently must obtain energy substrates from their surrounding environment. *In vivo* following ejaculation the required energy source may be obtained from seminal plasma and the secretions of the female reproductive tract, whereas *in vitro*, the energy supply must be present in the incubation media. A lack of energy supply will

undoubtedly interfere with the cell metabolism, suppressing such processes as motility and tyrosine phosphorylation, which could consequently compromise the function of the sperm cell.

The effect of the omission of albumin from the incubation media on tyrosine phosphorylation in rat epididymal spermatozoa was not as profound as that observed in mouse caudal epididymal spermatozoa (Visconti *et al.*, 1995a). Visconti *et al.*, 1995a demonstrated that the exclusion of BSA from the incubating media almost completely abolished the capacitation-associated changes in tyrosine phosphorylation. The situation demonstrated in the rat was intriguing, as a lack of albumin appeared to both positively and negatively affect the process of tyrosine phosphorylation in both the immature caput and mature caudal spermatozoa, in keeping with the suggestion made by Fraser (Fraser, 1985) in that albumin is not required for capacitation but for the acrosome reaction, which is often classified as the endpoint of capacitation. As tyrosine phosphorylation of proteins in mammalian spermatozoa, has been inextricably associated with the attainment of the capacitated state (Aitken *et al.*, 1998a; Aitken *et al.*, 1995; Visconti *et al.*, 1995a; Visconti *et al.*, 1995b), Fraser's explanation (Fraser, 1985) could possibly explain why the removal of albumin from the incubation media, did not exert a significantly deleterious effect on tyrosine phosphorylation in rat epididymal spermatozoa.

In conclusion, it is evident that the presence of both bicarbonate and glucose in the incubation media are vital for the *in vitro* support of tyrosine phosphorylation in both caput and caudal spermatozoa, whereas the requirement for albumin is not as absolute. In addition, the inhibitory consequences of bicarbonate exclusion from the incubation medium on tyrosine phosphorylation may be completely overcome by raising the pH of the extracellular environment, suggesting that the essential role of the anion is in the maintenance of an appropriate proton balance in the spermatozoon's cytoplasmic space.

Chapter Seven:

The role of calcium in the regulation of NADPH-induced ROS generation and tyrosine phosphorylation in rat epididymal spermatozoa

Chapter 7. The role of calcium in the regulation of NADPH-induced ROS generation and tyrosine phosphorylation in rat epididymal spermatozoa

7.1. Introduction

Calcium is generally believed to be one of the best known intracellular activators of sperm adenylate cyclase (Hyne and Garbers, 1979a; Kopf and Vacquier, 1984). However, this activation is dependent on the type of adenylyl cyclases, of which there are several. Calcium is known to stimulate the activity of type I, III and VIII adenylyl cyclases, but it actually inhibits types V and VI (Cooper *et al.*, 1995). It is thought that oscillations in cellular cAMP levels arise because of feedback inhibition of adenylyl cyclase by calcium (Cooper *et al.*, 1995).

It has been demonstrated in the mouse that calcium is crucial for the capacitation-associated changes in tyrosine phosphorylation as exemplified by the inhibitory effect of Ca^{2+}-free media on tyrosine phosphorylation and capacitation in the mouse (Visconti *et al.*, 1995b). Although calcium activates sperm adenylate cyclase (Hyne and Garbers, 1979a; Kopf and Vacquier, 1984), it is also known to stimulate the activity of a calmodulin dependent cyclic nucleotide phosphodiesterase, associated with demembranated rat caudal epididymal spermatozoa (Wasco and Orr, 1984) which could actually reduce [cAMP]i. In addition, Carrera *et al.*, 1996 found that calcium actually induced the dephosphorylation of human sperm proteins and this was calmodulin-dependent which suggested that calcineurin was involved. The initiation and maintenance of sperm motility is known to involve the cAMP-dependent phosphorylation of specific proteins (Lindemann and Kanous, 1989; San Agustin and Witman, 1994; Tash and Means, 1983). Sperm motility was also found to be inhibited by calcium and this was directly correlated with the inhibition of protein phosphorylation via the calmodulin-dependent protein phosphatase calcineurin (Tash *et al.*, 1988). However, it is also well established that the presence of calcium is crucial for the acrosome reaction as exemplified by the ability of the calcium ionophore A23187 to induce the acrosome reaction and increase sperm-oocyte fusion in human spermatozoa.

Evidently calcium plays a significant role in the regulation of motility, cAMP-mediated tyrosine phosphorylation, capacitation and the acrosome reaction in spermatozoa. Earlier chapters have already established that tyrosine phosphorylation is mediated by cAMP in the rat and is also potentially a redox-regulated process. Observations have also identified the differences associated with epididymal maturation in these processes. Bicarbonate has also been shown to influence both redox-cycling and tyrosine phosphorylation in rat epididymal spermatozoa *in vitro*. Similarly, as calcium is a crucial second messenger of various signal transduction cascades, it became clear that the role of this ion in the regulation of such processes was an important issue to address.

7.2. Materials and Methods

Refer to Chapter 2 for general materials and methods.

7.2.i. The effect of calcium free BWW (Ca^{2+}-free BWW) on NADPH-induced superoxide ($O_2^{-\cdot}$) generation in rat epididymal spermatozoa

As detailed in Section 2.6., detection of $O_2^{-\cdot}$ was measured by lucigenin-dependent chemiluminescence using Berthold luminometers (LB9505, Berthold Analytical Instruments, Wildbad, Germany) at 37°C. Lucigenin is a charged compound used for the detection of superoxide. It is relatively membrane impermeant and thus largely measures the release of production of $O_2^{-\cdot}$ to the extracellular space.

Normal BWW contains 1.7 mM $CaCl_2$, therefore to make Ca^{2+}-free BWW this compound was omitted from the media and replaced with NaCl. Rat spermatozoa were extracted from the epididymis as described in Section 2.3., except that sperm from one epididymis were released into normal BWW and those from the remaining organ released into Ca^{2+}-free BWW. Motility and density counts (Sections 2.4. and 2.5. respectively) were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. The procedure for detection of spontaneous $O_2^{-\cdot}$ generation by rat epididymal spermatozoa was carried out exactly as described in Section 2.6.i. When the addition of NADPH was required, the luminometer run was temporarily halted and NADPH diluted in BWW was added to the appropriate cuvettes, at a final concentration of 2 mM. Immediately following the addition of NADPH, the luminometer run was restarted. Both normal BWW and Ca^{2+}-free BWW without sperm were used as controls in each luminometer run.

7.2.ii The effect of Ca^{2+}-free BWW on tyrosine phosphorylation in epididymal rat spermatozoa

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one of the caput epididymis was diluted into normal BWW while those from the other were diluted straight into Ca^{2+}-free BWW. The same procedure was then carried out on the spermatozoa from the caudal region. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.). The spermatozoa were incubated with various treatments including 3 mM PTX, 2 mM NADPH and 5 mM dbcAMP for 3 hours at

37°C. On completion of the three hour incubation period, rat sperm proteins were extracted using SDS as described in Section 2.7. and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11.

In addition, slides were prepared from some of the sperm samples as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of tyrosine phosphorylated proteins. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14.

7.2.iii The effect of Ca^{2+}-free BWW on intracellular cAMP levels in epididymal rat spermatozoa

Following the release of rat spermatozoa into both normal BWW and Ca^{2+}-free BWW as described above in Sections 7.2.i. and 7.2.ii, motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. Spermatozoa were incubated at this concentration with either 3 mM PTX or the combined treatment of 3 mM PTX and 2 mM NADPH for 3 hours at 37°C. Following completion of the incubation period, extraction of cAMP from the spermatozoa was performed as described in Section 2.15.

7.2.iv The effect of phosphatase inhibitor Okadaic acid on tyrosine phosphorylation of rat epididymal spermatozoa

Okadaic acid inhibits type I and IIA protein phosphatases and it was incubated with epididymal rat spermatozoa in order to observe whether or not it exerted any inhibitory effects on tyrosine phosphorylation of sperm proteins.

The epididymides were separated into the caput and caudal regions as normal and each caput from the same animal was sectioned into 2 separate longitudinal parts. One piece of caput was placed into each of four petri dishes all containing 3 ml BWW. Of the four petri dishes, one contained normal BWW, the second BWW that had been supplemented with 10 nM Okadaic acid, the third BWW supplemented with 100 nM Okadaic acid and the fourth dish contained BWW supplemented with 1 μM Okadaic acid.

This was repeated with the caudal sections, motility and density counts were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. Cells were incubated for up to 3 hours incubation at 37°C.

Sperm extracts for Western Blot analysis of phosphorylated proteins were prepared following incubations of 30 min and 3 hours, in accordance with the guidelines set out in Sections 2.7-2.11. The detergent SDS was used for the extraction process.

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one of the caput epididymis was diluted into normal BWW while those from the other were diluted straight into BWW supplemented with 100 nM Okadaic acid. The same procedure was then carried out on the spermatozoa from the caudal region. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.). The spermatozoa were incubated with various treatments including 3 mM PTX and 5 mM dbcAMP for up to 5 hours at 37°C. Rat sperm proteins were extracted using SDS as described in Section 2.7. following 3 and 5 hours incubation and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11.

In addition, slides were prepared from some of the sperm samples as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of tyrosine phosphorylated proteins. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14.

7.2.v The effect of calcineurin inhibitor Cyclosporin A on tyrosine phosphorylation of rat epididymal spermatozoa

Calcineurin, a protein serine/threonine phosphatase (Martin, 1998), is unique in being the only Ca^{2+} and calmodulin-dependent protein phosphatase (Cohen, 1989; Klee *et al.*, 1988; Shenoliker and Nairn, 1990). Deltamethrin was used to determine the influence of calcineurin on tyrosine phosphorylation in a study on human sperm (Carrera *et al.*, 1996), as it was thought to be a potent inhibitor of this phosphatase (Enan and Matsumara, 1992). However this compound has since been proven, along with various other class II pyrethroid insecticides to be ineffective in the specific inhibition of calcineurin (Enz and Pombo-Villar, 1997; Fakata *et al.*, 1998b). Consequently Cyclosporin A was chosen for this experiment, primarily because along with FK 506, it is known to be a specific and potent inhibitor of calcineurin (Carballo *et al.*, 1999) and references therein.

The epididymides were separated into the caput and caudal regions as normal and each caput from the same animal was sectioned into three separate longitudinal parts. One piece of caput from both epididymides was placed into each of three petri dishes all containing 3 ml BWW. Consequently each petri dish should have contained two pieces of caput epididymis, one from each epididymis of the same animal. Of the three petri dishes, one contained normal BWW, the second contained BWW supplemented with 100 nM Cyclosporin A and the third BWW supplemented with 1 μM cyclosporin A.

This was repeated with the caudal sections, motility and density counts were performed and the concentration of each cell population adjusted to 10 x 10^6/ml. Cells were incubated for 3 hours incubation at 37°C with 3 mM PTX 2 mM NADPH and 5 mM dbcAMP. On completion of the incubation period, rat sperm proteins were extracted using SDS as described in Section 2.7. and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11.

7.2.vi Detection of calcineurin in protein extracts of rat epididymal spermatozoa

Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase and when active consists of a large calmodulin-binding catalytic subunit A (61 kDa) and a smaller Ca^{2+} binding subunit B (19 kDa). Following the release of rat spermatozoa into both normal BWW and Ca^{2+}-free BWW as described above in Sections 7.2.i. and 7.2.ii, motility and density counts were performed and the cell concentration adjusted to 10 x

10^6/ml. The spermatozoa were incubated with various treatments including 3 mM PTX, 2 mM NADPH and 5 mM dbcAMP for 3 hours at 37°C.

Rat sperm proteins were extracted using SDS as described in Section 2.7. following 3 hours incubation and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.). The proteins were analysed for the presence of calcineurin using the Western Blot protocol described in Section 2.11. except that the primary antibody PY20 was exchanged for a monoclonal antibody against Calcineurin (Affiniti, UK). This antibody was incubated with the nitrocellulose membranes for two hours at the manufacturers recommended dilution of 1 in 250, which equals a concentration of 20 µg per sheet of nitrocellulose.

7.2.vii Detection of phosphoserine and phosphothreonine in protein extracts of rat epididymal spermatozoa

The presence and possible involvement of serine/threonine protein kinases in human sperm cell capacitation and the acrosome reaction has previously been demonstrated (Naz *et al.*, 1993). In addition, the presence of a novel dual-specificity protein kinase of 42 kDa, capable of tyrosine and serine phosphorylation of specific proteins was identified in boar spermatozoa, thus raising the question of the involvement of its function in the protein kinase network mediating signal transduction in mammalian spermatozoa (Berruti, 1994). Thus it was of interest to establish the involvement of serine and threonine phosphorylation of proteins extracted from rat spermatozoa at different levels of epididymal maturation.

Following the release of rat spermatozoa into both normal BWW and Ca^{2+}-free BWW as described above in Sections 7.2.i. and 7.2.ii, motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. The spermatozoa were incubated with various treatments including 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C.

Rat sperm proteins were extracted using SDS as described in Section 2.7. following 3 hours incubation and duplicate protein samples separated according to molecular mass by SDS-PAGE (Section 2.9.). The proteins were analysed for the presence of phosphoserine and phosphothreonine using the Western Blot protocol described in Section 2.11. except that the primary antibody PY20 was exchanged for antibodies against phosphoserine and phosphothreonine. Two sets of antibodies were evaluated for this study. The first set were monoclonal antibodies to phosphothreonine and phosphoserine obtained from Affiniti, UK. Duplicate protein extracts were incubated for two hours at dilutions of 1 in 250, which equals a concentration of 16 µg

per sheet of nitrocellulose and 1 in 1000, which is equal to 4 μg per sheet of nitrocellulose. Only a small proportion of proteins are phosphorylated on either serine or threonine residues, consequently the manufacturers recommended at least 50 μg protein should be loaded per lane. This was in agreement with the minimum set for all the work detailed within this thesis (refer to Section 2.8.).

The second set of antibodies were polyclonal antibodies to phosphothreonine and phosphoserine obtained from Zymed Corporation, USA. They were also incubated at concentrations of 1 in 250 which equals a concentration of 40 μg per sheet of nitrocellulose and 1 in 1000, which is equal to 10 μg per sheet of nitrocellulose for the phosphothreonine antibody. The final concentration per sheet of nitrocellulose for the phosphoserine antibody was 20 μg and 5 μg respectively. The secondary antibody, Anti rabbit Ig HRP linked F $(ab')_2$ from donkey was incubated with the nitrocellulose at a dilution of 1 in 6000 for one hour as described in Section 2.11.

7.2.viii Detection of AKAP 220 in protein extracts of rat epididymal spermatozoa.

The type II cAMP-dependent PKA is compartmentalised in highly specific sites in the cell. In order to maintain their specific locations within the cell , the regulatory unit (RII) of type II PKAs interacts with RII-anchoring proteins. PKA type II anchoring proteins now form a family referred to as AKAP (A-Kinase Anchor Proteins). A testis-specific AKAP referred to as TAKAP-80 has previously been localised to the fibrous sheath of rat spermatozoa, whose presence was found to be particularly evident in mature animals (Mei *et al.*, 1997). AKAP 220 is a protein of 1129 amino acids and its mRNA was found expressed ubiquitously with the highest levels in testis and brain.

Following the release of rat spermatozoa into both normal BWW and Ca^{2+}-free BWW as described above in Sections 7.2.i. and 7.2.ii, motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. The spermatozoa were incubated with various treatments including 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C.

Rat sperm proteins were extracted using SDS as described in Section 2.7. following 3 hours incubation and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.). The proteins were analysed for the presence of AKAP 220 using the Western Blot protocol described in Section 2.11. except that the primary antibody PY20 was exchanged for a monoclonal antibody against AKAP 220 (Affiniti, UK). This antibody was incubated with the ntrocellulose membranes for two hours at

the manufacturers recommended dilution of 1 in 250, which equals a concentration of 20 μg per sheet of nitrocellulose.

In addition, slides were prepared from some of the sperm samples as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of AKAP 220. The protocol used was identical to that described in Section 2.14. except that PY20 was replaced with antibody to the AKAP 220 which was added at the same concentration as PY20. Negative controls were set up by replacing AKAP 220 with normal mouse serum as described in Section 2.14.

7.2.ix Intracellular calcium measurements in rat epididymal spermatozoa

Rat epididymal spermatozoa were extracted from the epididymis and released into BWW as described in Section 2.3. Motilities and densities were performed and the cells adjusted to concentrations of 10 x 10^6/ml (refer to Sections 2.4. and 2.5.). Intracellular Ca^{2+} levels in rat spermatozoa were evaluated as described in Section 2.16.

7.2.x Tyrosine phosphorylation in ejaculated rat spermatozoa obtained from the female uterus

A single male rat was placed in a wire-bottomed cage with a single female rat overnight. The purpose of the wire-bottomed cages was so that when the vaginal plug that signifies that copulation has taken place is evacuated from the female tract, it is easily detected in the container beneath the wire bottom. The animals were maintained within the temperature range of 20-25°C and relative humidity was kept between 45 to 70%. The animals were fed with Rat and Mouse No. 1 Expanded Diet (Special Diets Services Ltd) and water from the domestic supply *ad libitum*. The next morning the cages were checked for vaginal plugs and the females were sacrificed as described in Section 2.2., if it was evident that they had mated with the males.

The lower abdomen of the female was incised and the ovaries and uterine horns located using watchmakers forceps. Surgical thread was then tied around the base of the uterus just before it branched into the right and left horn. Thread was also tied around the apical region of each horn, immediately adjacent to the ovary and using fine scissors the uterine horns were removed from the pelvic cavity of the carcass. The thread was removed and using forceps to hold the base, the uterus was held over a test tube. A sterile syringe containing prewarmed BWW was then inserted into the base of the uterus and the media was gently injected into the organ to flush any sperm and fluid

out of the uterine horns. The organ was discarded and the extract checked under the microscope for the presence of sperm. The motility and density of sperm was counted as described in Sections 2.4 and 2.5. and sperm proteins were extracted using SDS as described in Section 2.7. The supernatant was retained after the first centrifugation and the fluid centrifuged a further 2 times at 700 g for 6 minutes at 25°C to ensure that the last remains of any spermatozoa were removed. The sperm proteins were separated according to molecular mass by SDS-PAGE (Section 2.9.), followed by analysis for tyrosine phosphorylation by Western Blot as described in Section 2.11. SDS-PAGE and Western Blot analysis was also carried out the retained female tract fluid to provide an internal control.

In addition, slides were prepared from some of the sperm samples as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of tyrosine phosphorylated proteins. Negative controls were set up by replacing PY20 with normal mouse serum as described in Section 2.14.

7.3. Results

7.3.i. The effect of calcium free BWW (Ca^{2+}-free BWW) on NADPH-induced superoxide ($O_2^{-\cdot}$) generation in rat epididymal spermatozoa

The requirement for Ca^{2+} in capacitating media and the zona pellucida induced acrosome reaction has been well established in many species (Yanagimachi, 1994). Visconti *et al.*, 1995b, also demonstrated the necessity for Ca^{2+} in incubation media for the capacitation related appearance of phosphotyrosine-containing proteins in the mouse. However, as demonstrated in previous chapters, the physical likeness between the mouse and rat does not predispose them to similarities in the regulation of sperm function. The effect of Ca^{2+} on $O_2^{-\cdot}$ generation and tyrosine phosphorylation in rat spermatozoa proved to be unique in itself.

Interestingly, the removal of calcium from the incubation medium led to the inhibition of spontaneous $O_2^{-\cdot}$ generation, although these levels were not statistically significant. The effect of Ca^{2+}-free BWW on NADPH induced $O_2^{-\cdot}$ generation in both caput and caudal rat spermatozoa was interesting in that production was up-regulated in both caput and caudal spermatozoa under these conditions (refer to Fig. 7.1. and 7.2.). As found previously, the $O_2^{-\cdot}$ burst induced following the addition of NADPH was significantly greater in spermatozoa obtained from the caput region, in comparison with those from the cauda epididymis irrespective of the different incubation media (Fig. 7.1. and 7.2.). When compared with rat epididymal spermatozoa in normal BWW, caput and caudal spermatozoa incubated in Ca^{2+}-free conditions consistently generated significantly increased $O_2^{-\cdot}$ in response to NADPH (Fig. 7.1. and 7.2.).

Figure 7.1. The effect of Ca^{2+}-free BWW on 2 mM NADPH-induced lucigenin-dependent chemiluminescent $O_2^{\cdot-}$ generation in both caput and caudal spermatozoa. 2 mM NADPH was added to both complete BWW and Ca^{2+}-free BWW for use as controls (n = 4).

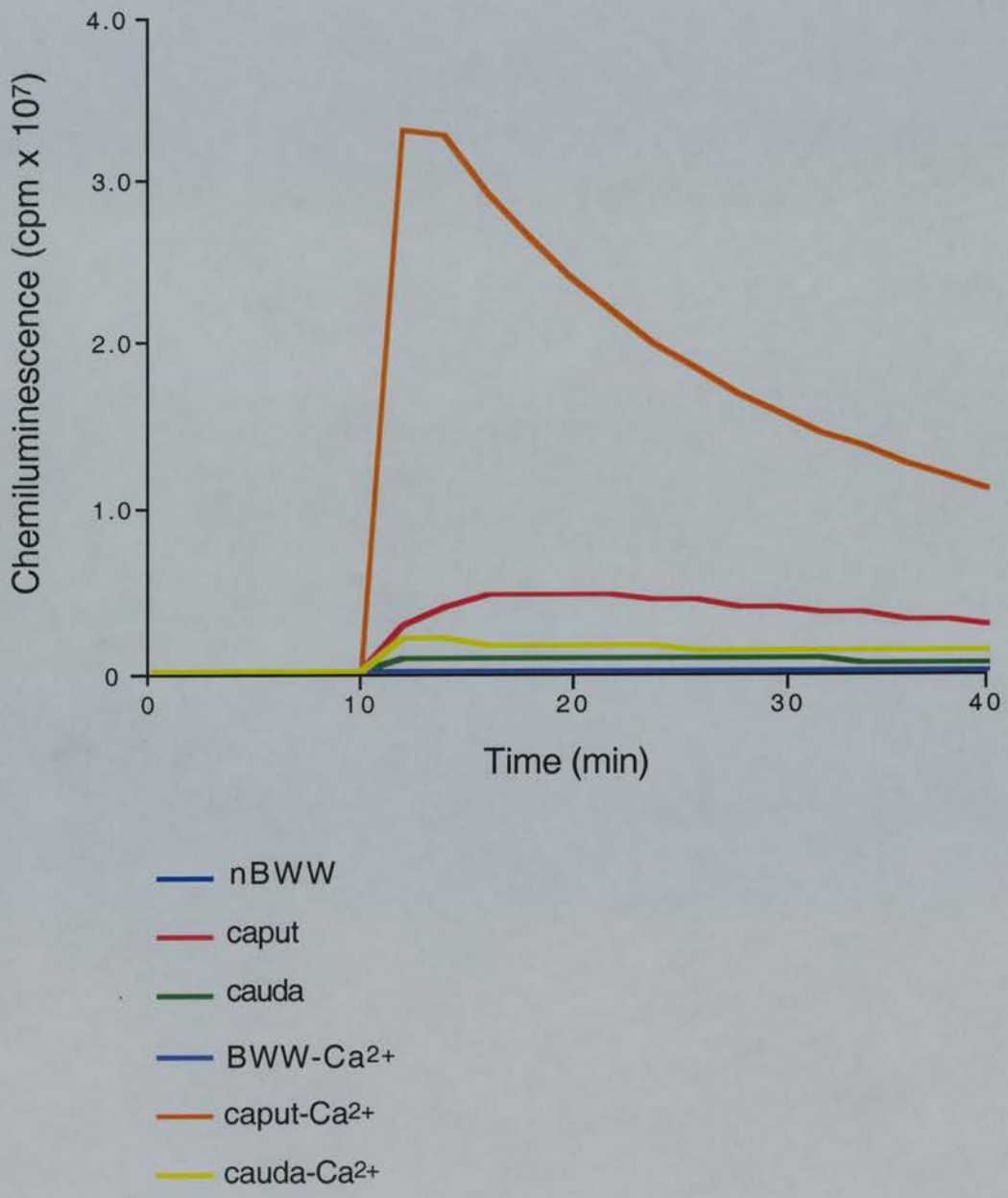
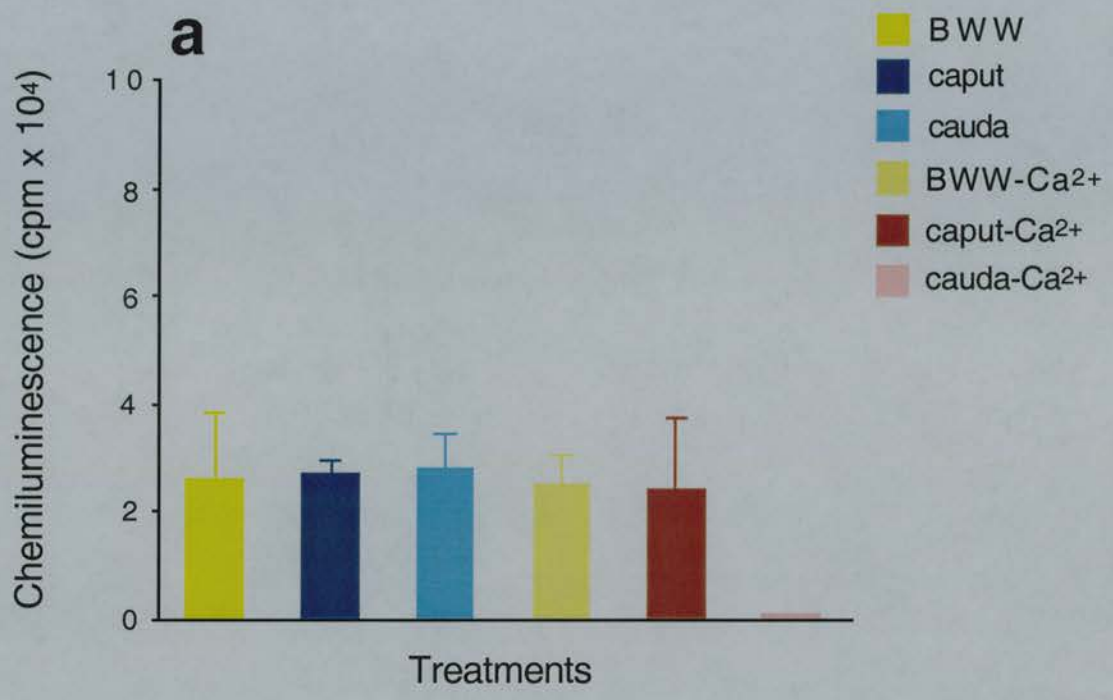
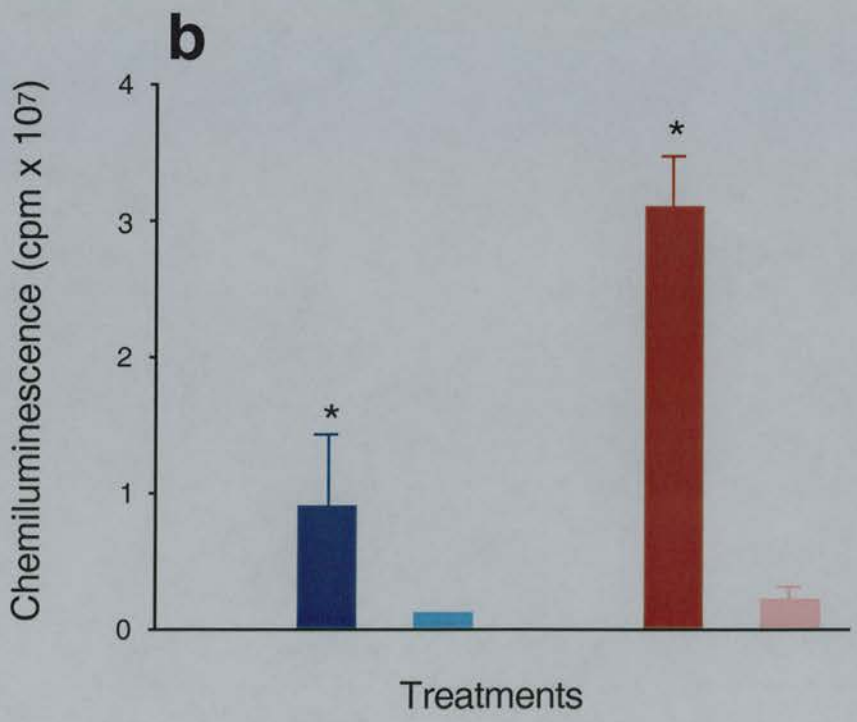
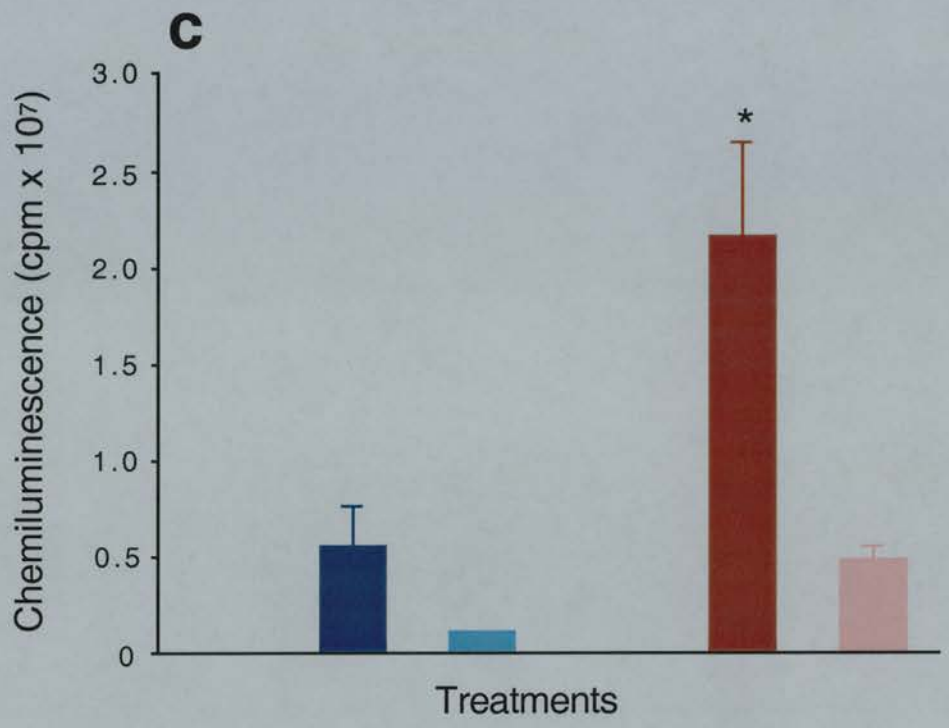
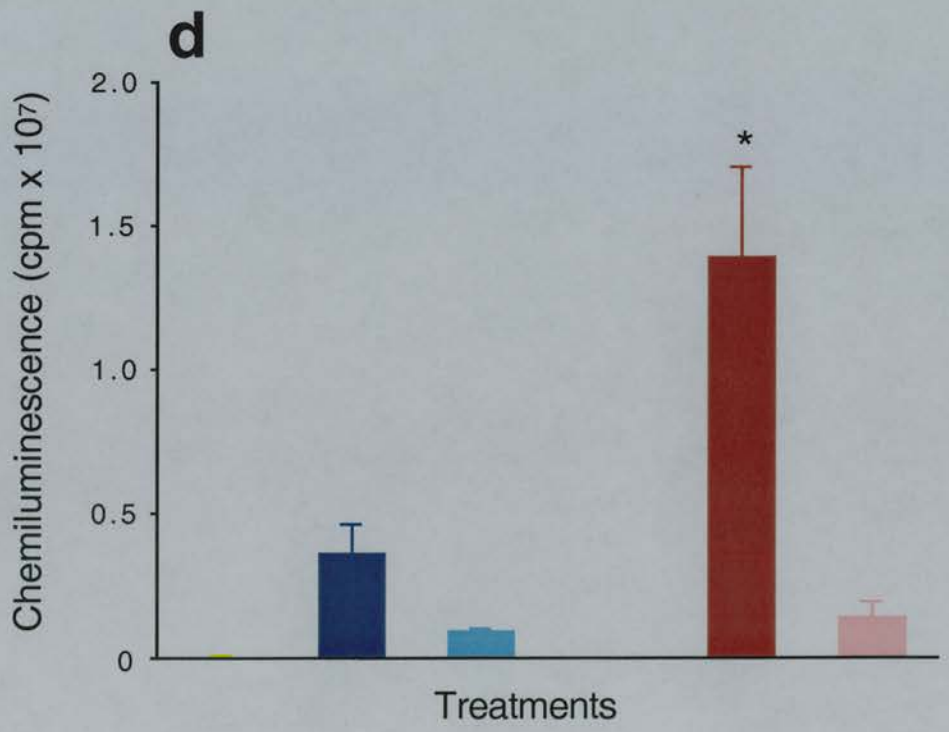

Chemiluminescence (cpm x 10⁷)
4.0
3.0
2.0
1.0
0
0
10
20
30
40
Time (min)
nBWW
caput
cauda
BWW-Ca2+
caput-Ca2+
cauda-Ca2+

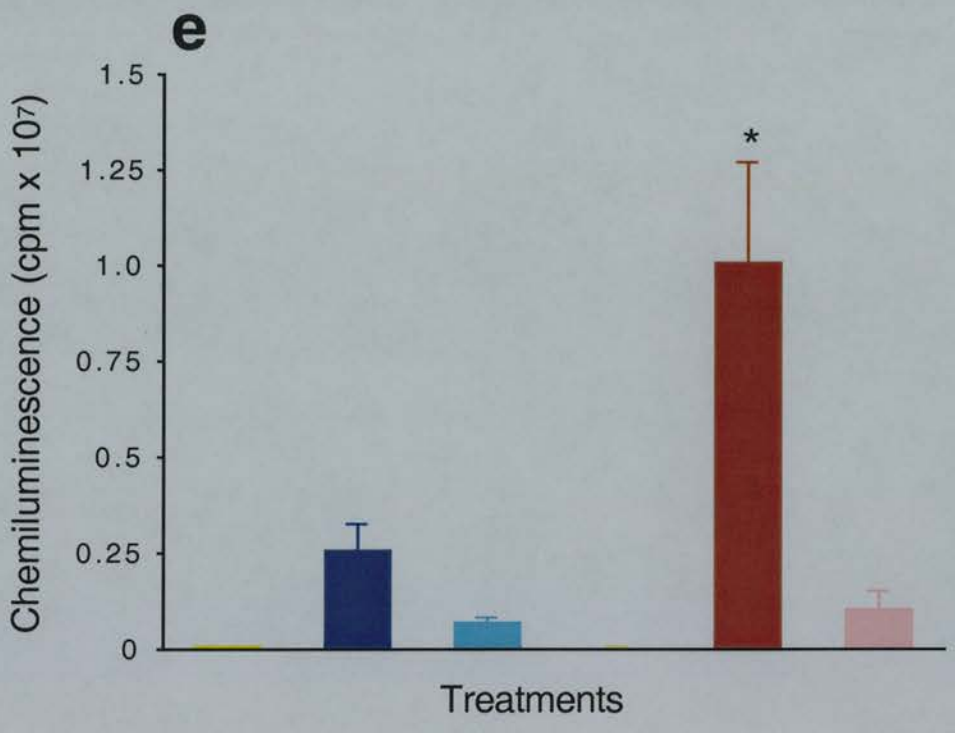
Figure 7.2. The following bar charts represent the mean production of $O_2^{\cdot-}$ in response to NADPH in rat spermatozoa, incubated in both complete BWW and Ca^{2+}-free BWW. Superoxide induced chemiluminescence was measured in counts per minute (cpm) following **(a)** 0 minutes, **(b)** 12 minutes, **(c)** 20 minutes, **(d)** 30 minutes and **(e)** 40 minutes incubation (n = 4).


a
BWW
caput
cauda
BWW-Ca2+
caput-Ca2+
cauda-Ca2+
Chemiluminescence (cpm x 10⁴)
10
8
6
4
2
0
Treatments


b
Chemiluminescence (cpm x 10⁷)
4
3
2
1
0
*
*
Treatments


c
3.0
2.5
2.0
1.5
1.0
0.5
0
Chemiluminescence (cpm x 10⁷)
*
Treatments


d
2.0
1.5
1.0
0.5
0
Chemiluminescence (cpm x 10⁷)
*
Treatments


e
Chemiluminescence (cpm x 10⁷)
1.5
1.25
1.0
0.75
0.5
0.25
0
*
Treatments

7.3.ii The effect of Ca^{2+}-free BWW on tyrosine phosphorylation in epididymal rat spermatozoa

As in the $NaHCO_3^-$-free experiment, epididymides from one animal were used in the same experiment and spermatozoa from one organ were released into complete BWW and spermatozoa from the other organ were released into Ca^{2+}-free BWW.

In complete contrast to the mouse (Visconti *et al.*, 1995a) the removal of $CaCl_2$ from the BWW media actually up-regulated tyrosine phosphorylation in both caput and caudal spermatozoa (Fig. 7.3.). Western Blot analysis demonstrated that in all treatments including the controls, phosphorylation was induced in numerous proteins and also intensified in proteins already phosphorylated, although the stimulating effect was particularly exaggerated in spermatozoa from the cauda epididymis (Fig. 7.3.). These results are in agreement with the observations of Carrera *et al* (Carrera *et al.*, 1996), whereby it was found in human spermatozoa that phosphorylation was reduced when sperm were incubated either in increasing concentrations of extracellular Ca^{2+}, or in a medium containing the Ca^{2+} ionophore A23187.

Immunocytochemistry demonstrated that the increase in phosphorylation was localised to the tail in caput spermatozoa as well as in the caudal spermatozoa (Fig. 7.4. and 7.5.). This was of particular interest as explained in Chapter 4, protein phosphorylation had not been able to be induced in the tail of caput spermatozoa, even following stimulation with the active cAMP analogue, dbcAMP. Spermatozoa were counted to establish the percentage population of cells displaying positive staining for phosphotyrosine-containing proteins in the tail (Fig. 7.5.). It was clear that Ca^{2+}-free conditions significantly increased the number of cells demonstrating tyrosine phosphorylation in the tail following all treatments (Fig. 7.5.). Caput cells treated with a combination of 3 mM PTX and 5 mM dbcAMP increased the tail phosphorylated population from less than 15% in complete BWW to approximately 80% in Ca^{2+}-free BWW (Fig. 7.5.). Additionally the control caput cells increased their positive tail stained population from approximately 1% in complete BWW to over 20% in Ca^{2+}-free conditions (Fig. 7.5.). The effect of Ca^{2+}-free conditions on caudal spermatozoa was equally significant with the phosphorylated tail population in the controls increasing from less than 5% under normal conditions, to approximately 55% in Ca^{2+}-free BWW (Fig. 7.5.).

Figure 7.3. The effect of Ca^{2+} on tyrosine phosphorylation in rat epididymal spermatozoa. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either BWW or Ca^{2+}-free BWW, at 37°C for 3 hours with the following treatments: 3 mM PTX; 3 mM PTX + 5 mM dbcAMP; 3 mM PTX + 2 mM NADPH (n = >5).

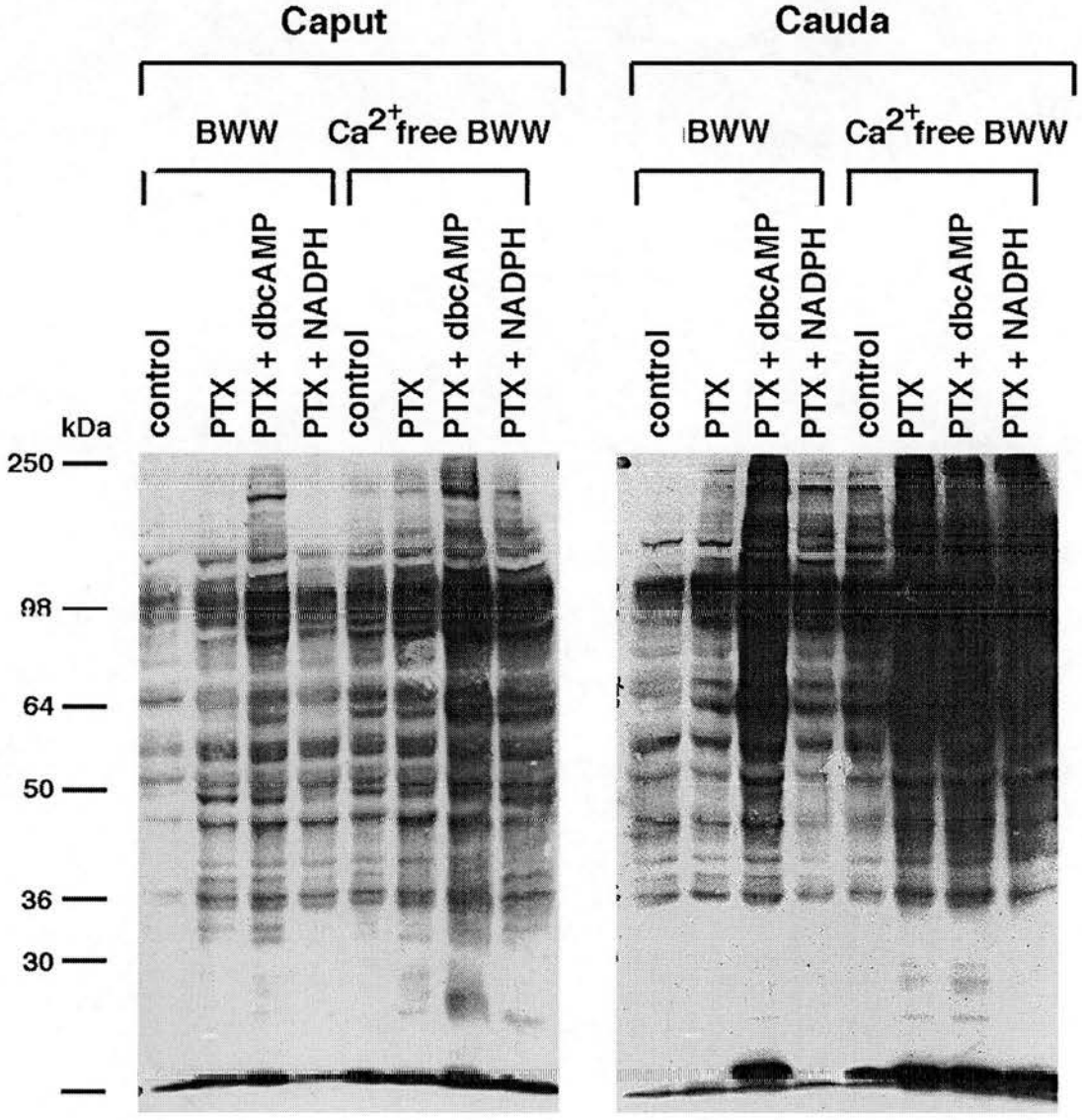

Caput
Cauda
BWW
Ca2+free BWW
BWW
Ca2+free BWW
control
PTX
PTX + dbcAMP
PTX + NADPH
control
PTX
PTX + dbcAMP
PTX + NADPH
control
PTX
PTX + dbcAMP
PTX + NADPH
control
PTX
PTX + dbcAMP
PTX + NADPH
kDa
250
98
64
50
36
30

Figure 7.4. Immunolocalisation of tyrosine phosphorylated proteins in caput and caudal spermatozoa fixed with 1% paraformaldehyde following incubation in either BWW or Ca^{2+}-free BWW, at 37°C for 3 hours with 3 mM PTX + 2 mM NADPH (n = 4) (magnification x 400):

(i) Caput spermatozoa in BWW with 3 mM PTX + 2 mM NADPH.

(ii) Caput spermatozoa in Ca^{2+}-free BWW with 3 mM PTX + 2 mM NADPH.

(iii) Caput spermatozoa in BWW with 3 mM PTX + 2 mM NADPH (negative controls).

(iv) Caput spermatozoa in Ca^{2+}-free BWW with 3 mM PTX + 2 mM NADPH (negative controls).

(v) Cauda spermatozoa in BWW with 3 mM PTX + 2 mM NADPH.

(vi) Cauda spermatozoa in Ca^{2+}-free BWW with 3 mM PTX + 2 mM NADPH.

(vii) Cauda spermatozoa in BWW with 3 mM PTX + 2 mM NADPH (negative controls).

(viii) Cauda spermatozoa in Ca^{2+}-free BWW with 3 mM PTX + 2 mM NADPH (negative controls).

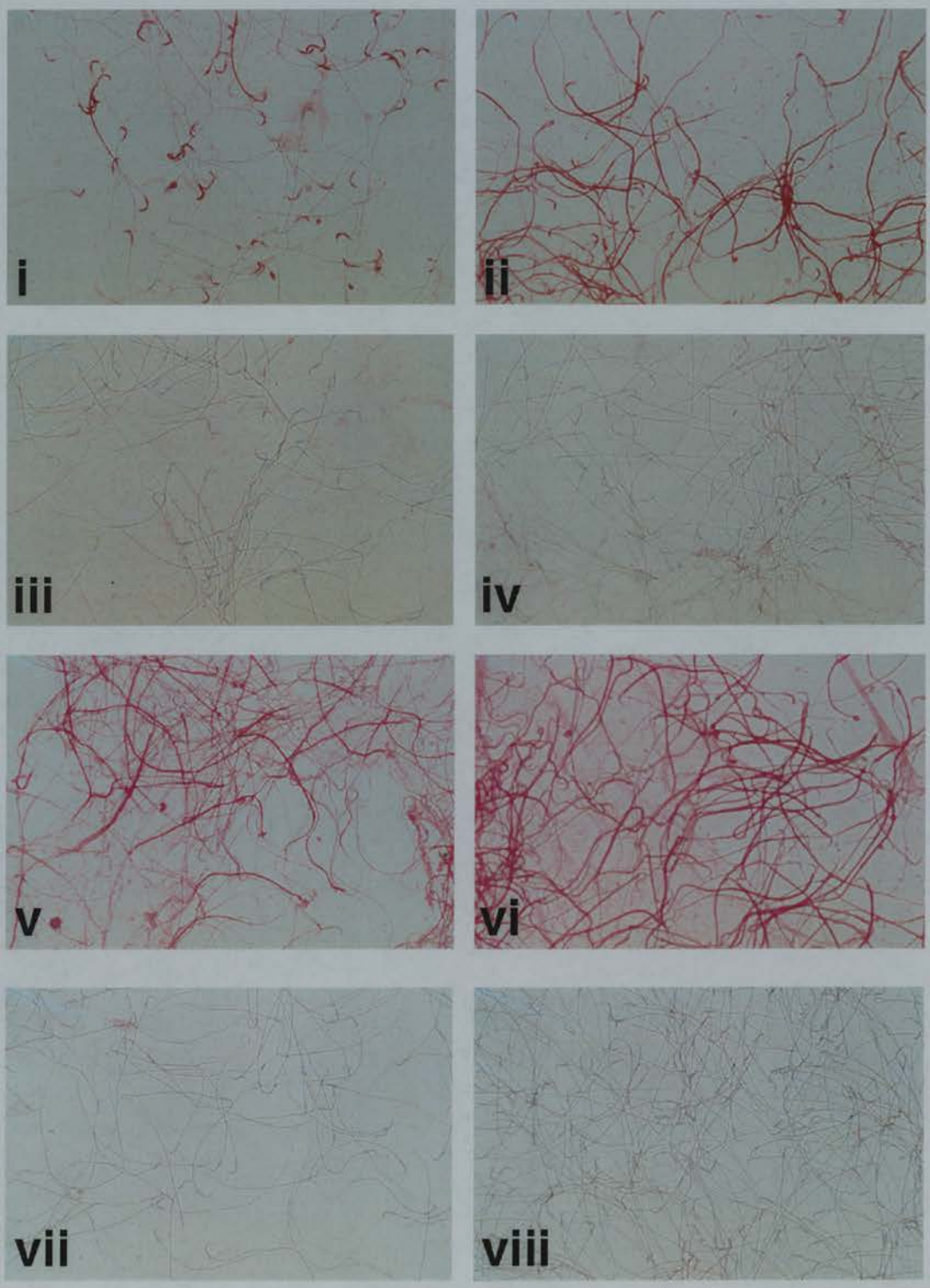
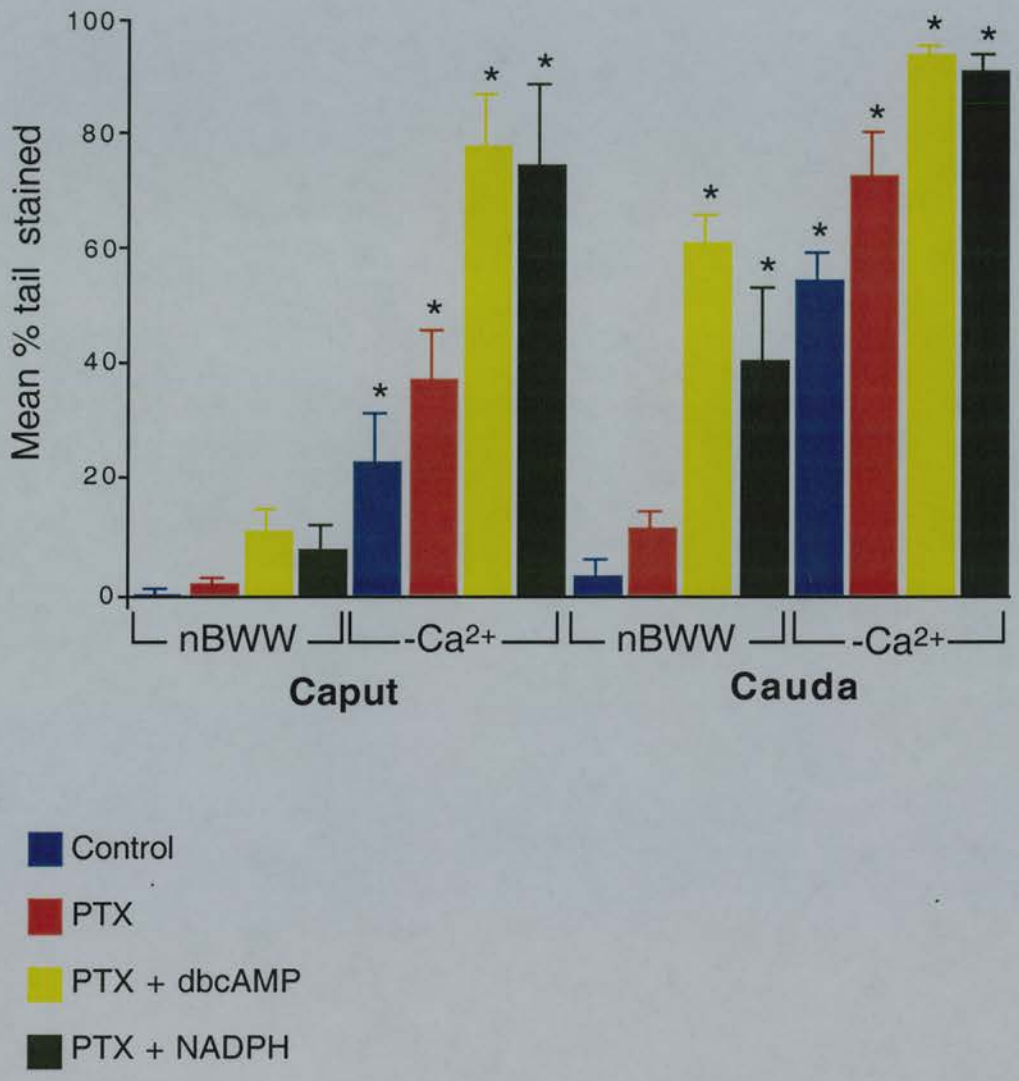

i
ii
iii
iv
v
vi
vii
viii

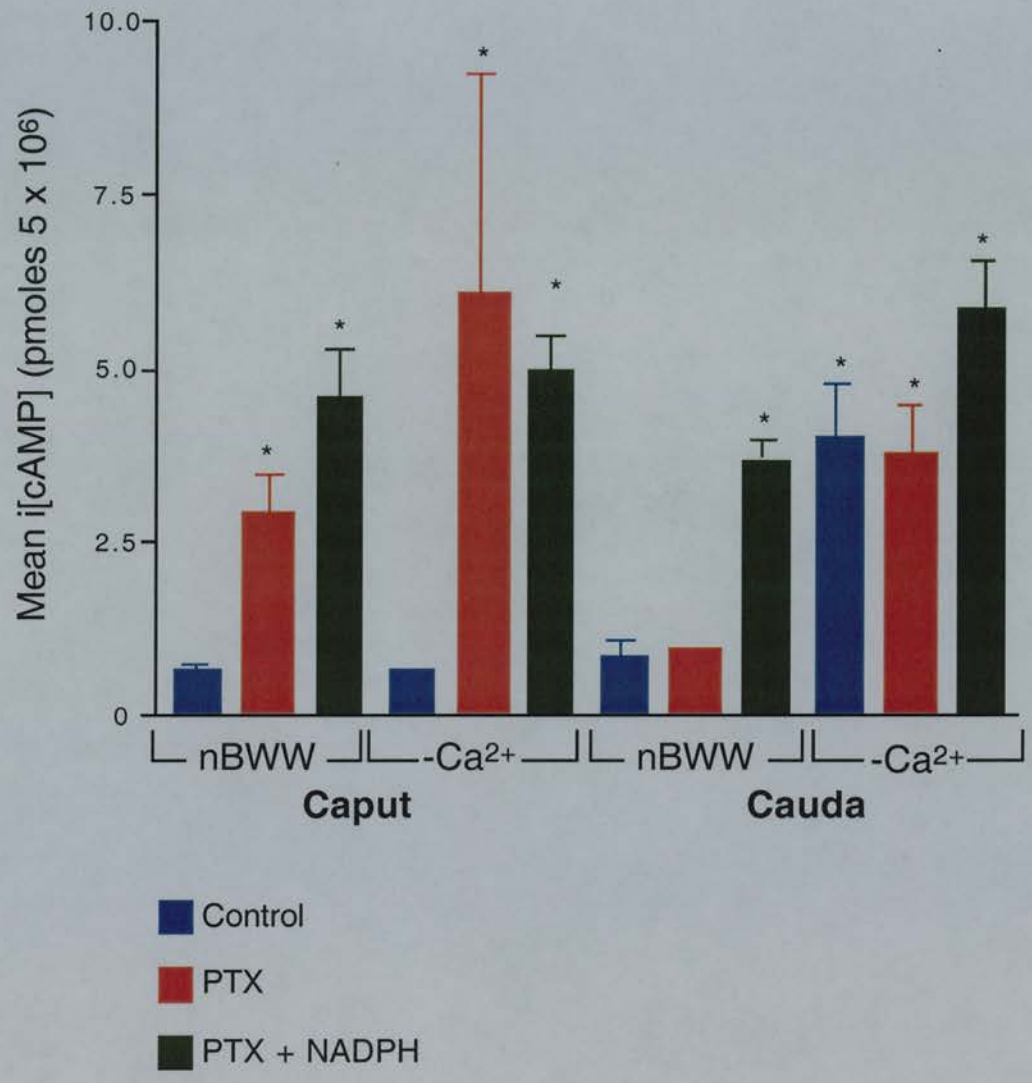
Figure 7.5. Graphical representation of the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation in the tail, following incubation in either nBWW or Ca^{2+}-free BWW, at 37°C for 3 hours with the following treatments: 3 mM PTX; 3 mM PTX + 5 mM dbcAMP; 3 mM PTX + 2 mM NADPH (n = 4).


Mean % tail stained
100
80
60
40
20
0
nBWW
-Ca2+
nBWW
-Ca2+
Caput
Cauda
Control
PTX
PTX + dbcAMP
PTX + NADPH

7.3.iii The effect of Ca^{2+}-free BWW on intracellular cAMP levels in epididymal rat spermatozoa

Ca^{2+}-free conditions did increase [cAMP]i in rat spermatozoa but this was not statistically significant in all treatments. In caput spermatozoa, Ca^{2+}-free conditions only significantly increased treated [cAMP]i in cells that had been incubated with 3 mM PTX (Fig. 7.6.). However, in caudal spermatozoa, the only treatment in Ca^{2+}-free conditions that did not have significantly higher [cAMP]i than its corresponding treatment in normal BWW were spermatozoa that had been incubated in 3 mM PTX with 2 mM NADPH.

Figure 7.6. Data representing the mean intracellular concentration of cAMP in caput and caudal spermatozoa incubated in either BWW or Ca^{2+}-free BWW, for 3 hours at 37°C with the following treatments: 3 mM PTX; 3 mM PTX + 2 mM NADPH (n = 6).


Mean i[cAMP] (pmoles 5 x 10⁶)
10.0
7.5
5.0
2.5
0
nBWW
-Ca2+
nBWW
-Ca2+
Caput
Cauda
Control
PTX
PTX + NADPH

7.3.iv The effect of phosphatase inhibitor Okadaic acid on tyrosine phosphorylation of rat epididymal spermatozoa

Cells must be able to dephosphorylate proteins phosphorylated by PKA as often the effects of cAMP should be transient (Alberts *et al.*, 1989). The dephosphorylation of phosphorylated serines and threonines is catalysed by four groups of serine/threonine phosphoprotein phosphatases: protein phosphatases I, IIA, IIB and IIC (Alberts *et al.*, 1989). All of these phosphatases (except for IIC) are composed of a homologous catalytic subunit complexed with one or more regulatory subunits (Alberts *et al.*, 1989). Whereas protein phosphatase-I plays an important role in the response to cAMP, IIA has a broad specificity and appears to be primarily responsible for reversing many of the phosphorylations catalysed by serine/threonine kinases (Alberts *et al.*, 1989). Protein phosphatase-IIB, also referred to as calcineurin is activated by Ca^{2+}.

As tyrosine residues are thought to be phosphorylated via a PKA dependent tyrosine kinase, it was logical to try and determine the influence of such phosphatases on the regulation of this process in rat epididymal spermatozoa. Consequently rat spermatozoa were incubated with Okadaic acid, a potent inhibitor of type I and IIA protein phosphatases.

Figure 7.7a. demonstrates that the optimum concentration of Okadaic acid for inducing up-regulation of tyrosine phosphorylation in both caput and caudal spermatozoa was 100 nM. Consequently, this dose was chosen to further evaluate the effect of this compound on this process. As described in Section 7.2.iv., spermatozoa were incubated in either normal BWW or BWW supplemented with 100 nM Okadaic acid for 3 - 5 hours. Following 3 hours incubation, caput spermatozoa incubated with okadaic acid demonstrated increased phosphorylation of proteins of approximately 50 and 98-130 kDa, in comparison with the corresponding control caput cells incubated in normal BWW (Fig. 7.7b.). Caput spermatozoa stimulated with PTX and dbcAMP, suspended in BWW supplemented with Okadaic acid exhibited phosphorylation of a novel protein of approximately 150 kDa, although phosphorylation of a 200 kDa protein was actually down-regulated under these conditions (Fig. 7.7b.). Untreated caudal spermatozoa incubated in Okadaic acid demonstrated increased levels of tyrosine phosphorylation in proteins of approximately 98-115 kDa, when compared with the untreated caudal controls in normal BWW. There was no difference between caudal spermatozoa treated with PTX and dbcAMP incubated in either normal BWW or media supplemented with Okadaic acid (Fig. 7.7b.).

Following 5 hours incubation, the intensity of phosphorylation had decreased in untreated caput spermatozoa, irrespective of the type of media they had been incubated

in (Fig. 7.7b,c.). However, in comparison with the caput cells treated with PTX and dbcAMP for 3 hours, those incubated for 5 hours exhibited phosphorylation of a novel protein of approximately 180 kDa in addition to the up-regulated phosphorylation of a protein of 115 kDa (Fig. 7.7b,c.). There was no real difference in the levels of tyrosine phosphorylation between those cAMP stimulated caput cells incubated in normal BWW and those in the supplemented media (Fig. 7.7b,c.).

In contrast caudal control spermatozoa exhibited the phosphorylation of two new proteins of 115 and 180 kDa, both of which were not phosphorylated in control spermatozoa following 3 hours incubation (Fig. 7.7b,c.). The intensity of phosphorylation was slightly increased in those cells incubated in the supplemented media (Fig. 7.7b,c.). However, as demonstrated following the shorter incubation, there was no difference in phosphorylation of tyrosine residues on sperm proteins in the cAMP stimulated caudal spermatozoa, irrespective of the incubation media (Fig. 7.7b,c.).

When rat spermatozoa were subjected to immunocytochemical protocol following exposure to the various treatments, it was clear that the changing patterns in phosphorylation demonstrated by Western Blot analysis, were not reflective of changes in the phosphorylation of tail proteins (Fig. 7.8.). Neither was the reduction in phosphorylation of caput sperm proteins following 5 hours incubation, correlated with a reduction in positive staining of proteins located to the acrosomal region (data not shown).

Figure 7.7. The effect of phosphatase inhibitor, okadaic acid on tyrosine phosphorylation in rat epididymal spermatozoa. **(a)** A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated at 37°C for 3 hours with the following treatments (n = 2):

(i) Control

(ii) 10 nM okadaic acid

(iii) 100 nM okadaic acid

(iv) 1 μM okadaic acid

(b) and **(c)** Western Blots of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated at 37°C for 3 **(b)** and 5 **(c)** hours with the following treatments (n = 5):

(i) Caput control/ nBWW

(ii) Caput 3mM PTX + 5 mM dbcAMP/ nBWW

(iii) Cauda control/ nBWW

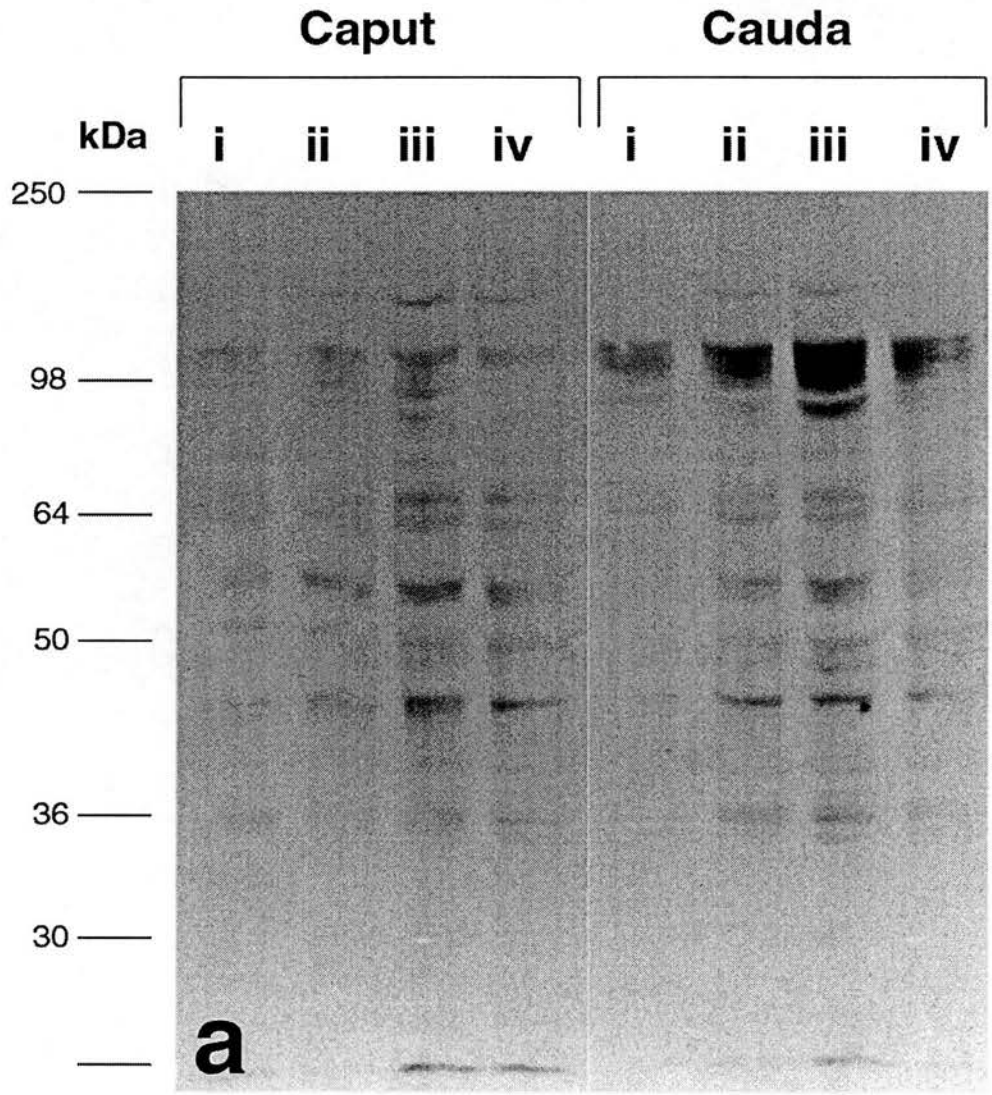
(iv) Cauda 3mM PTX + 5 mM dbcAMP/ nBWW

(v) Caput control/ 100 nM okadaic acid

(vi) Caput 3mM PTX + 5 mM dbcAMP/ 100 nM okadaic acid

(vii) Cauda control/ 100 nM okadaic acid

(viii) Cauda 3mM PTX + 5 mM dbcAMP/ 100 nM okadaic acid


Caput
Cauda
kDa
i
ii
iii
iv
i
ii
iii
iv
250
98
64
50
36
30
a

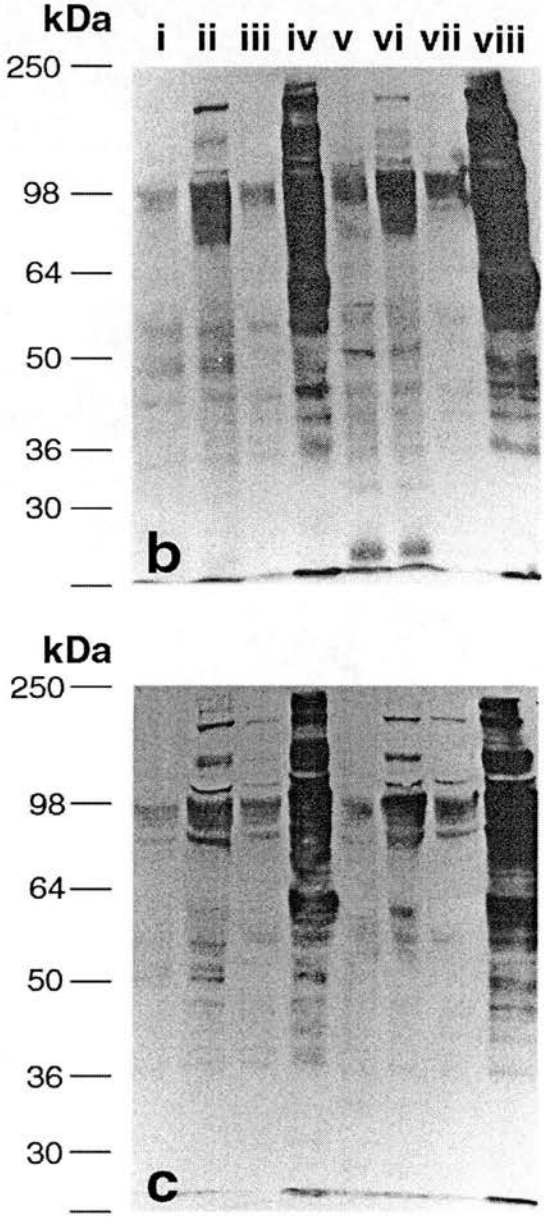
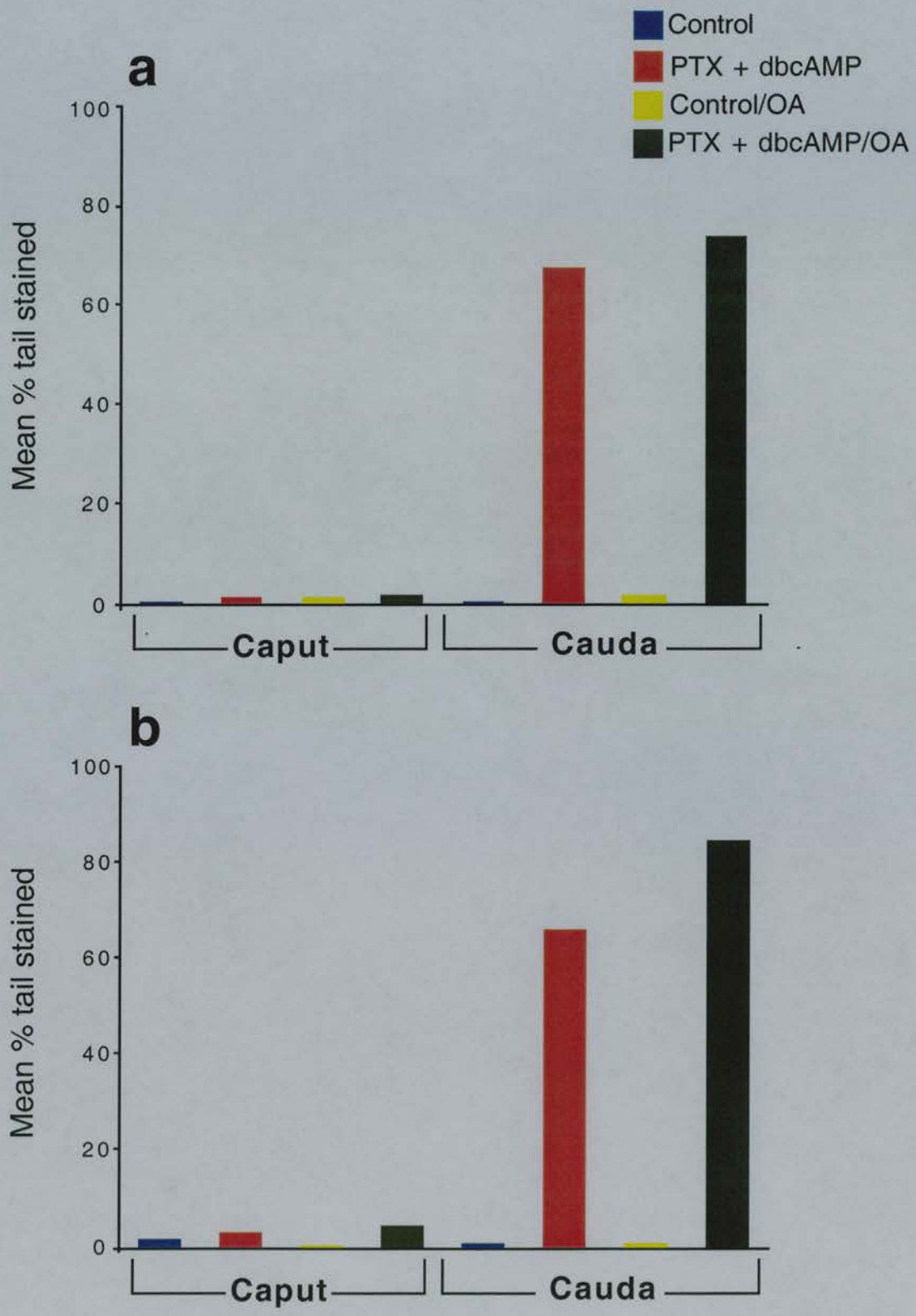

kDa
i ii iii iv v vi vii viii
250
98
64
50
36
30
b
kDa
250
98
64
50
36
30
c

Figure 7.8. Graphical representation of the percentage population of caput and caudal spermatozoa exhibiting positive staining for tyrosine phosphorylation in the tail. The cells had been incubated in either nBWW or BWW supplemented with 100 nM okadaic acid, at 37°C for (a) 3 hours and (b) 5 hours with the following treatments: control and 3 mM PTX + 5 mM dbcAMP (n = 2).


a
Control
PTX + dbcAMP
Control/OA
PTX + dbcAMP/OA
Mean % tail stained
100
80
60
40
20
0
Caput
Cauda
b
Mean % tail stained
100
80
60
40
20
0
Caput
Cauda

7.3.v The effect of calcineurin inhibitor Cyclosporin A on tyrosine phosphorylation of rat epididymal spermatozoa

Cyclosporin A appeared to exert very little effect on tyrosine phosphorylation of rat epididymal spermatozoa (Fig.7.9.). In caput spermatozoa, that had previously been incubated with PTX and dbcAMP, there appeared to be a slight dose-dependent up-regulation of tyrosine phosphorylation in the higher molecular weight proteins of approximately 135, 180 and 200 kDa. However, Cyclosporin A appeared to exert no effect on caudal spermatozoa except in those that had been treated with PTX and NADPH whereby a slight dose-dependent increase in tyrosine phosphorylation was observed (Fig. 7.9.).

7.3.vi Detection of calcineurin in protein extracts of rat epididymal spermatozoa.

The presence of calcineurin in rat epididymal sperm extracts was not evident when analysed by Western Blot analysis (Fig. 7.10.). However, the results obtained were inconsistent in that sometimes many non-specific protein bands were detected and other times as shown in Figure 7.10., no proteins at all were detected even following exposures of 15-30 minutes.

Figure 7.9. The effect of calcineurin inhibitor, Cyclosporin A on tyrosine phosphorylation in rat epididymal spermatozoa. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either nBWW, BWW supplemented with 100 nM cyclosporin A or BWW supplemented with 1 μM cyclosporin A at 37°C for 3 hours with the following treatments (n =3):

(a) Caput **(b)** Cauda

(i) Control

(ii) 3 mM PTX + 2 mM NADPH

(iii) 3 mM PTX + 5 mM dbcAMP

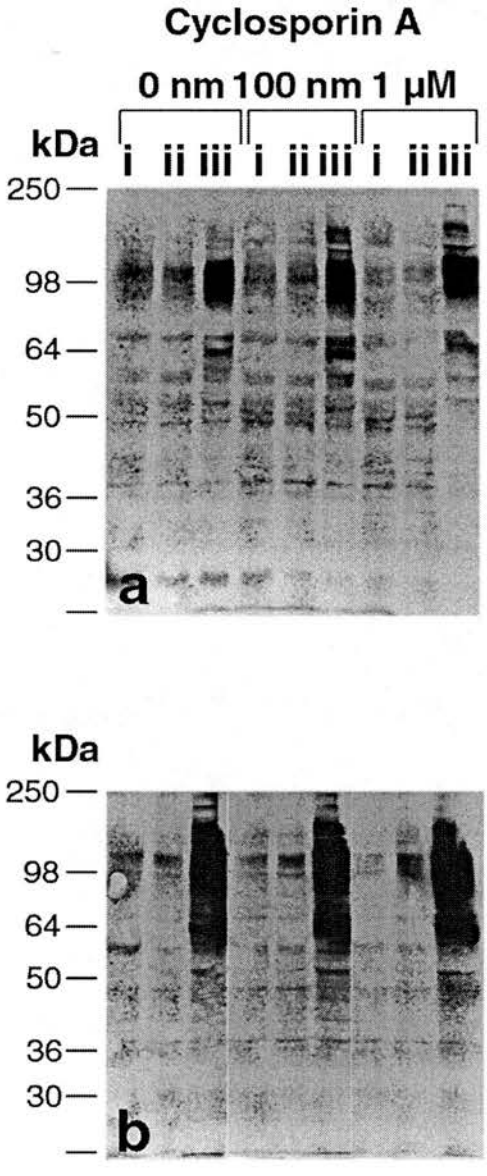

Cyclosporin A
0 nm 100 nm 1 µM
kDa
i ii iii i ii iii i ii iii
250
98
64
50
36
30
a
kDa
250
98
64
50
36
30
b

Figure 7.10. Detection of calcineurin in rat epididymal spermatozoa. A Western Blot of rat sperm epididymal proteins probed with a monoclonal antibody against calcineurin following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either BWW or Ca^{2+}-free BWW, at 37°C for 3 hours with the following treatments (n = 5):

(i) Control/ nBWW

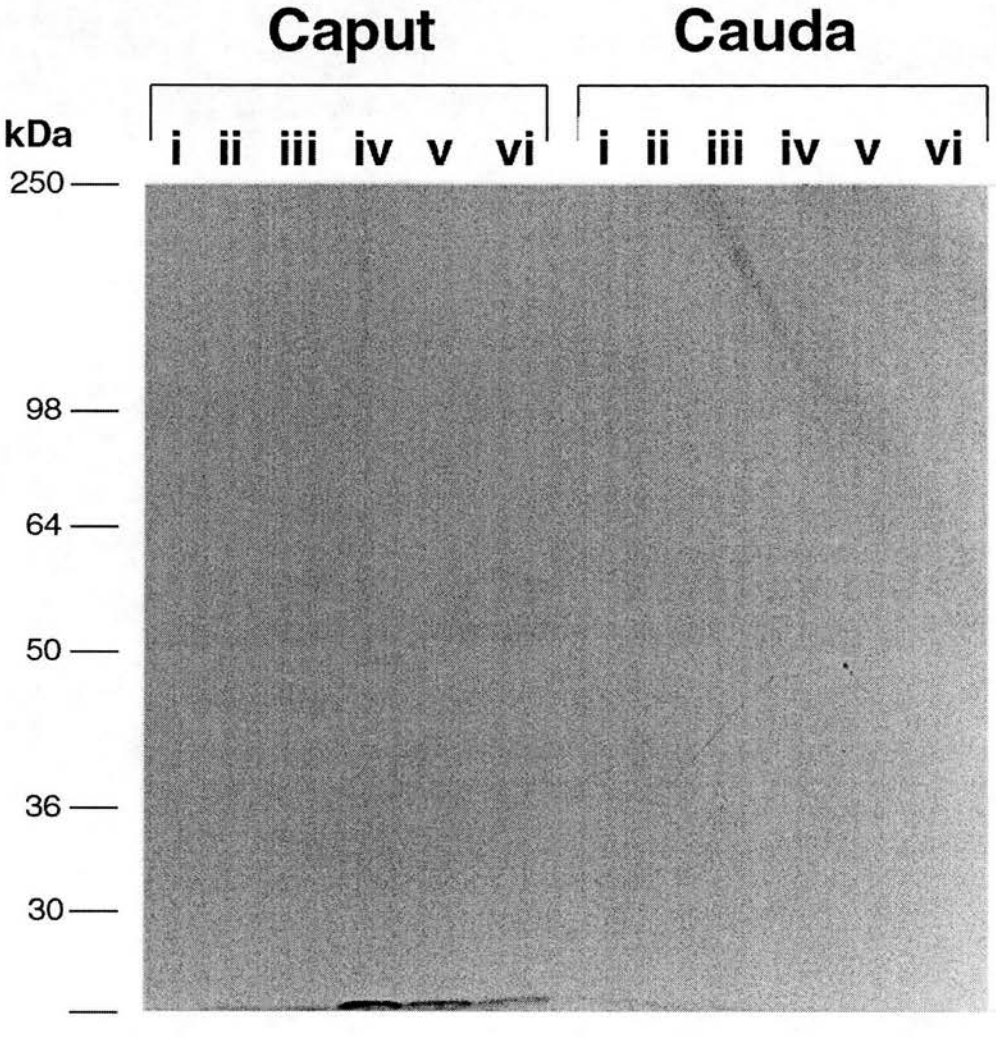
(ii) 3 mM PTX + 5 mM dbcAMP/ nBWW

(iii) 3 mM PTX + 2 mM NADPH/ nBWW

(iv) Control/ Ca^{2+}-free BWW

(v) 3 mM PTX + 5 mM dbcAMP/ Ca^{2+}-free BWW

(vi) 3 mM PTX + 2 mM NADPH/ Ca^{2+}-free BWW


Caput
Cauda
kDa
i ii iii iv v vi
i ii iii iv v vi
250
98
64
50
36
30

7.2.vii Detection of phosphoserine and phosphothreonine in protein extracts of rat epididymal spermatozoa

Phosphorylation of both serine and threonine residues on proteins failed to be detected, irrespective of the variable conditions (data not shown). There was either no positive band at all as found with the Zymed Corporation antibodies, or overexposure of the film to the nitrocellulose membrane was evident even after exposures as short as 5 seconds, when the Affiniti antibodies were used (data not shown). Unfortunately it was not possible to optimise the use of any of the tested antibodies for detection of phosphorylated serine and threonine residues.

7.3.viii Detection of AKAP 220 in protein extracts of rat epididymal spermatozoa.

Western Blot analysis provided no indication of the presence of AKAP 220 in rat epididymal spermatozoa irrespective of the organ region from which they had been obtained (Fig. 7.11a.). Therefore it is unlikely that the protein of approximately 225 kDa, identified in previous chapters following stimulation of intracellular cAMP could be AKAP 220 (Fig. 7.11.). However, immunocytochemical analysis did demonstrate the presence of AKAP 220 in the lower tail region including the fibrous sheath in approximately 6% of the population of caudal spermatozoa that had been incubated in Ca^{2+}-free BWW with PTX and dbcAMP (Fig.7.11b-e.).

7.3.ix Intracellular calcium measurements in rat epididymal spermatozoa

The results obtained suggested that there was no significant difference in intracellular calcium levels between spermatozoa that had been extracted from either the caput or cauda epididymis (Fig. 7.12.).

Figure 7.11. Detection of AKAP 220 in rat epididymal spermatozoa. **(a)** A Western Blot of rat sperm epididymal proteins probed with a monoclonal antibody against AKAP 220 following extraction from caput and caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either BWW or Ca^{2+}-free BWW, at 37°C for 3 hours with the following treatments (n = 1):

(i) Control/ nBWW

(ii) 3 mM PTX + 5 mM dbcAMP/ nBWW

(iii) Control/ Ca^{2+}-free BWW

(iv) 3 mM PTX + 5 mM dbcAMP/ Ca^{2+}-free BWW

(b)-(d) Immunolocalisation of AKAP 220 proteins in caudal spermatozoa fixed with 1% paraformaldehyde following incubation in BWW at 37°C for 3 hours (n = 1) (magnification x 1000).

(e) Negative control

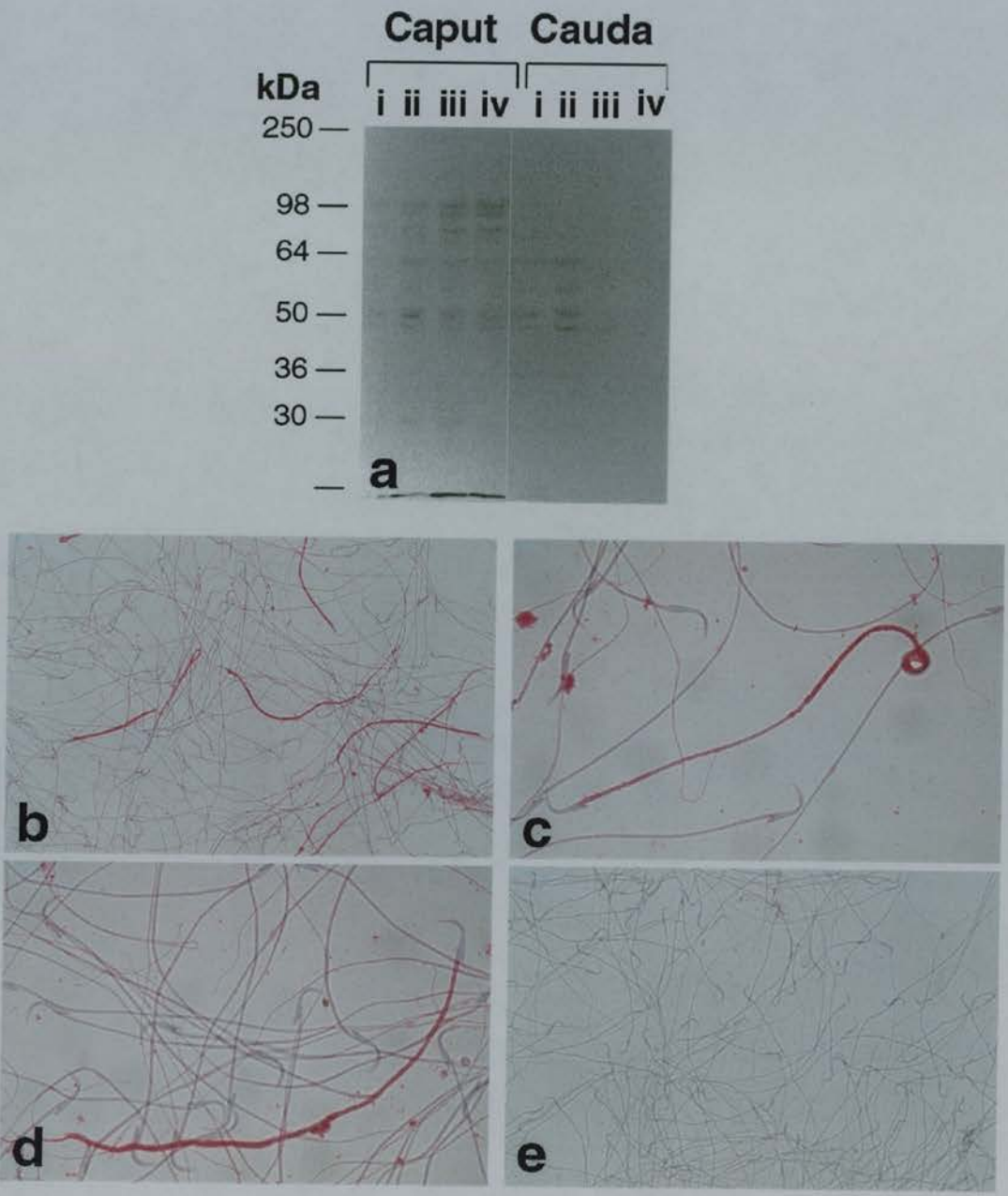
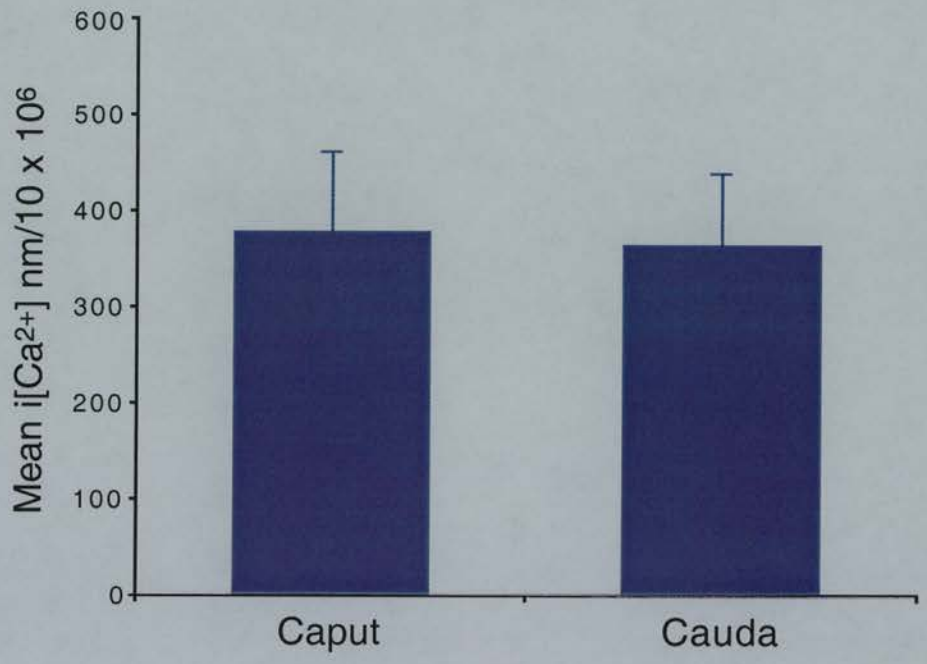

Caput
Cauda
kDa
i ii iii iv i ii iii iv
250
98
64
50
36
30
a
b
c
d
e

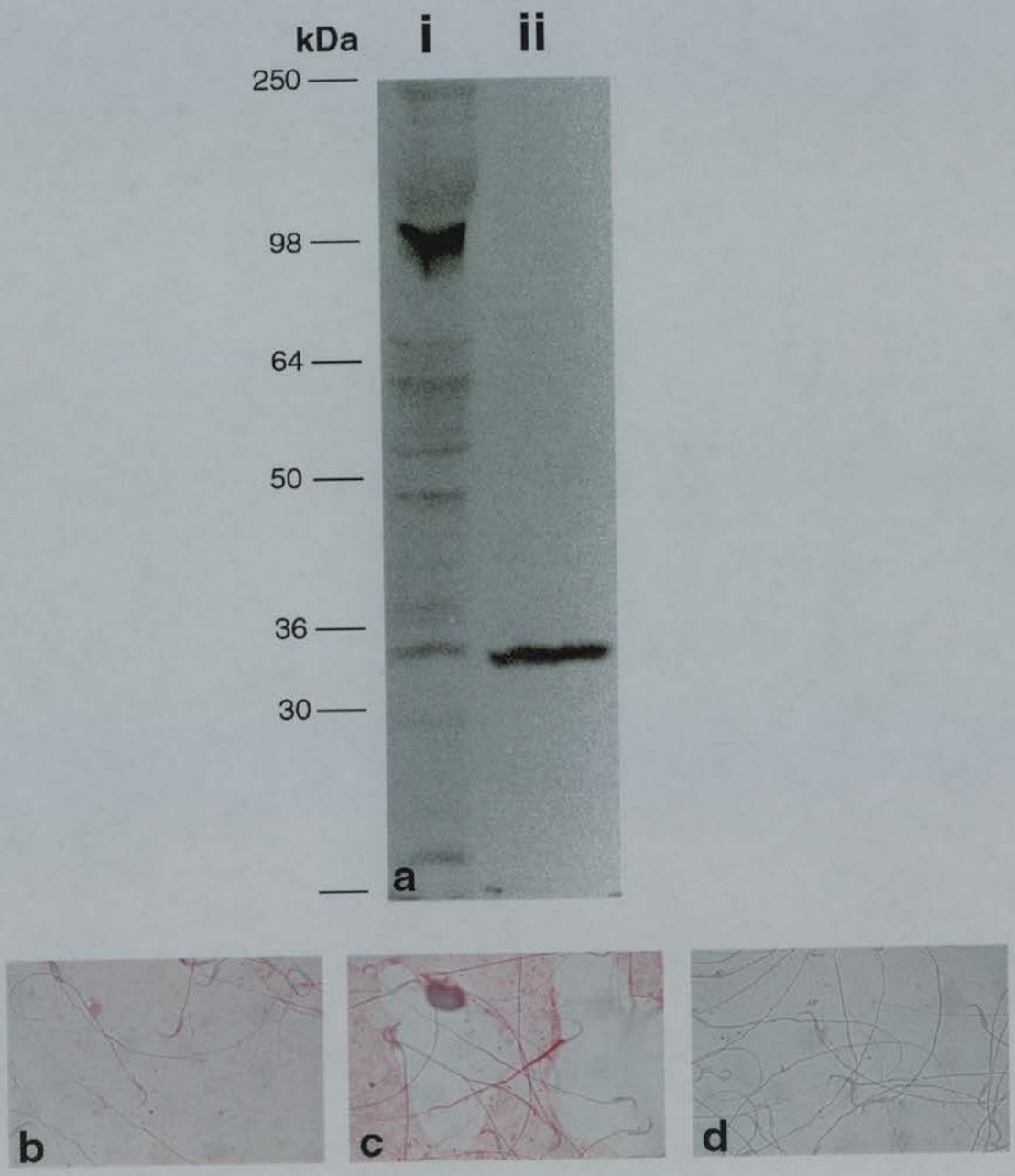
Figure 7.12. Data representing the mean intracellular concentration of Ca^{2+} in caput and caudal spermatozoa suspended in BWW (n = 8).


Mean i[Ca2+] nm/10 x 10⁶
600
500
400
300
200
100
0
Caput
Cauda

7.3.x Tyrosine phosphorylation in ejaculated rat spermatozoa obtained from the female uterus

The results demonstrated phosphorylation of multiple proteins in ejaculated spermatozoa obtained from the rat uterus following mating (Fig. 7.13a.). A protein of approximately 98 kDa was heavily phosphorylated and a protein of approximately 225 kDa was also phosphorylated (Fig. 7.13a.). Only one low molecular weight protein was detected in the female uterine tract fluid, confirming the source of the detected phosphorylated proteins to be the spermatozoa flushed from the uterus (Fig. 7.13a.). Unfortunately, the experiment was difficult to repeat as facilities to change the light and dark cycle and thus the time of mating were not available. Consequently this lead to an inconsistent yield of sperm from the female tract and often the sperm were dead. Staining of the posterior acrosomal domain could be observed in many of the spermatozoa, which was similar to the pattern observed in caudal spermatozoa. Tail phosphorylation was also observed following immunocytochemical analysis, however this type of staining was only identified in a few cells (Fig. 7.13b-d.).

Figure 7.13. **(a)** A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction with 1% SDS from ejaculated spermatozoa obtained from the uterus post-mating (n = 1). **(i)** Proteins extracted from ejaculated spermatozoa. **(ii)** Proteins extracted from female reproductive tract fluid (negative control).
(b)-(c) Immunocytochemical localisation of tyrosine phosphorylated proteins in ejaculated spermatozoa obtained from the uterus following copulation. **(d)** Negative control (magnification x 1000).


kDa
i
ii
250
98
64
50
36
30
a
b
c
d

7.4. Discussion

It is broadly accepted in the scientific community that the presence of Ca^{2+} is crucial for the successful capacitation of mammalian spermatozoa and the zona pellucida induced acrosome reaction (Yanagimachi, 1994). Difficulties with the current definition of capacitation are exemplified by the ability of the calcium ionophore, A23187 to induce an almost immediate acrosome reaction, presumably because of its ability to transport Ca^{2+} (Garbers and Kopf, 1980). However, capacitation can also occur in the absence of calcium and it is the acrosome reaction that is generally considered to be dependent on extracellular Ca^{2+} (Garbers and Kopf, 1980).

In the mouse Ca^{2+} is important for the capacitation-associated appearance of tyrosine phosphorylated proteins (Visconti *et al.*, 1995a). In contrast, Carrera *et al* (Carrera *et al.*, 1996) observed in human spermatozoa that Ca^{2+} in the extracellular media led to concentration dependent reductions in tyrosine phosphorylation. In this study of the rat, when $CaCl_2$ was excluded from the BWW, all the biochemical attributes of capacitation, including $O_2^{\cdot-}$ generation, tyrosine phosphorylation and [cAMP]i were enhanced in contrast to the results obtained when cells were incubated in $NaHCO_3^-$-free BWW (refer to Chapter 6.).

Ca^{2+}-free BWW induced an increased NADPH-induced $O_2^{\cdot-}$ response in both caput and caudal spermatozoa. This was unexpected, as activation of neutrophil oxidases, including NADPH oxidase is thought to be Ca^{2+} dependent (Kim-Park *et al.*, 1997). In the thyroid gland NADPH-dependent generation of H_2O_2 required Ca^{2+} (Deme *et al.*, 1985) although the NADPH-dependent H_2O_2-generating enzyme from thyroid particles could be active in the absence of Ca^{2+} (Dupuy *et al.*, 1988). The PMA-induced respiratory burst in neutrophils was inhibited following chelation of extracellular Ca^{2+} with EGTA, but it was potentiated when intracellular Ca^{2+} was chelated with BAPTA, suggesting that the protein kinase C (PKC)-mediated burst may be diminished by intracellular Ca^{2+} dependent phosphatase (Kim-Park *et al.*, 1997). It is possible that by incubating rat spermatozoa in Ca^{2+}-free conditions, intracellular Ca^{2+} levels are disrupted which may affect Ca^{2+} dependent phosphatases, consequently leading to an increased $O_2^{\cdot-}$ response.

Tyrosine phosphorylation in both caput and caudal spermatozoa was significantly increased under Ca^{2+}-free conditions. Of particular interest was the fact that tyrosine phosphorylation of the tail was induced in caput spermatozoa, which had not previously been demonstrated even following stimulation of intracellular cAMP levels with PTX and dbcAMP. The initiation and maintenance of motility may be linked with the cAMP-dependent phosphorylation of proteins (Lindemann and

Kanous, 1989; Tash and Means, 1983) and there is also a correlation between Ca^{2+} inhibited motility and suppression of tyrosine phosphorylation via the calmodulin-dependent protein phosphatase, calcineurin (phosphatase IIB) (Tash *et al.*, 1988). Carrera *et al* (Carrera *et al.*, 1996) found in human spermatozoa that when calcium or A23187, the calcium ionophore, were added to the incubation medium, tyrosine phosphorylation was inhibited. However, calmidazolium, the calmodulin antagonist, inhibited the A23187-induced negative effect on tyrosine phosphorylation, indicating the importance of both Ca^{2+} and calmodulin in the regulation of protein phosphorylation levels (Carrera *et al.*, 1996). It is possible that a phosphatase such as calcineurin is involved in the inhibition of tyrosine phosphorylation in rat spermatozoa, particularly the immature caput spermatozoa, as by simply withdrawing extracellular Ca^{2+}, a mature, caudal-like, tail dominated, tyrosine phosphorylation pattern was observed in a high proportion of the sperm population.

Carrera *et al* (Carrera *et al.*, 1996) also found that Okadaic acid, a potent inhibitor of phosphatases I and IIA, but a weak calcineurin inhibitor and deltamethrin, the calcineurin inhibitor, both inhibited the A23187-induced dephosphorylation of human spermatozoa (Carrera *et al.*, 1996). Consequently it was concluded that tyrosine phosphorylation in human spermatozoa may be partly regulated by calcineurin (Carrera *et al.*, 1996). The effect of Okadaic acid on tyrosine phosphorylation in rat spermatozoa was not as profound as observed in the human. Western Blot analysis demonstrated an increase in tyrosine phosphorylation of several proteins: however, the phosphorylation of tyrosine residues of tail proteins was not increased in either caput or caudal spermatozoa. These data indicate the involvement of phosphatases other than phosphatases I an IIA.

However, it is difficult to convincingly determine the involvement of calcineurin as the traditionally used inhibitors, such as the pyrethroid insecticides, deltamethrin, cypermethrin and fenvalerate are deemed to be ineffective inhibitors of calcineurin and other phosphatases (Fakata *et al.*, 1998a) (Enz and Pombo-Villar, 1997a). Therefore the data presented by Carrera *et al* 1996 indicating the involvement of calcineurin in the regulation of tyrosine phosphorylation are perhaps not as conclusive as originally thought. Cyclosporin A and FK506 are thought to be more potent inhibitors of calcineurin (Carballo *et al.*, 1999), but the nature of these compounds makes it difficult to incubate them with live cells as the viability of the cells is likely to be compromised. Cyclosporin A is difficult to maintain in aqueous solution. Therefore an important factor that must be taken into consideration in regards to this work is the amount of ethanol introduced as a vehicle for this compound. Spermatozoa lose viability in increasing concentrations of ethanol. Consequently it was important that Cyclosporin

A was diluted in a low volume of ethanol to allow a suitable ethanol to BWW ratio for the incubation of the cells. This together with the difficulty of maintaining Cyclosporin A in aqueous BWW, lead to the restriction of having to incubate spermatozoa with low concentrations of the inhibitor. Cyclosporin A appeared to have no effect on tyrosine phosphorylation levels of rat spermatozoa. This could be due either to the low incubation concentration of the inhibitor, or more likely as a result of the compound coming out of solution as following addition to the BWW media its presence did induce a slight cloudiness to the otherwise clear solution. Cyclosporin A also has low potency as a calcineurin inhibitor in cells expressing high levels of P-glycoprotein (Fakata *et al.*, 1998c). Consequently due to the unsuitability of the conditions, the influence of calcineurin activity in the regulation of tyrosine phosphorylation of rat spermatozoa cannot be ruled out, although it must be kept in mind that calcineurin failed to be detected in rat spermatozoa by Western Blot analysis.

These results suggest the presence of either a calcium-dependent tyrosine kinase inhibitor or protein phosphatase. The data indicates the likelihood that it is a calcium-dependent phosphatase as caput spermatozoa have relatively high unstimulated levels of tyrosine phosphorylation, located to the head. Therefore, tyrosine kinases must be active otherwise all tyrosine phosphorylation would be inhibited rather than just in the tail region. This raises the possibility of the presence of a calcium-dependent phosphatase located in the tail region of rat spermatozoa, but it does not explain why tail phosphorylation is only inhibited in caput spermatozoa. The activity levels of this proposed calcium-dependent phosphatase may differ depending on the epididymal region, or it could be that the membranes of caput spermatozoa are more permeable than caudal cells which could lead to increased intracellular calcium levels, thus leading to the increased activity of calcium-dependent phosphatases.

Although it was clearly demonstrated in hamster epididymal spermatozoa that unstimulated intracellular calcium levels were significantly higher in spermatozoa obtained from the caput region than those obtained from the caudal region (White and Aitken, 1989), this observation was not emulated in the rat. Thus, although intracellular Ca^{2+} levels were greater in rat caput spermatozoa than caudal cells, the difference was not statistically significant. Although there does not appear to be a significant difference in intracellular Ca^{2+} levels in the two types of cell, these results do not investigate the availability of Ca^{2+} in the epididymis itself as the cells were removed from the organ in order to be assessed. It is possible that the increased permeable nature of immature caput spermatozoa could lead to greater movement of

Ca^{2+} from within the cell to outwith the cell and vice versa, leading to fluctuations in intracellular concentration.

Hydrogen peroxide has been proven to inhibit calcineurin activity in human neutrophils (Carballo *et al.*, 1999). This raises the possibility that H_2O_2 production in caudal spermatozoa provides a mechanism for calcineurin inhibition present only in mature cells, as spontaneous H_2O_2 levels in caput spermatozoa are insignificant.

In Ca^{2+}-free conditions, [cAMP]i was increased in rat spermatozoa, particularly in those from the caudal region. There is evidence of a calmodulin-dependent cyclic nucleotide phosphodiesterase associated with demembranated rat caudal epididymal sperm (Wasco and Orr, 1984). The phosphodiesterase was stimulated two-fold in the presence of Ca^{2+} and the simultaneous addition of Ca^{2+} and calmodulin resulted in a four-fold increase in activity (Wasco and Orr, 1984). The presence of such an enzyme could explain why Ca^{2+}-free conditions induced an elevation in [cAMP]i, which was particularly evident in the caudal spermatozoa. By removing calcium from the extracellular media, the activity of the phosphodiesterase would be compromised.

Although the conditions for the mating experiment were not ideal, it was of interest to observe the phosphorylation of the 225 kDa protein in ejaculated spermatozoa obtained from the uterus in addition to a protein of 98 kDa. Both these proteins are phosphorylated in caudal spermatozoa following stimulation of their intracellular cAMP levels with either NADPH or dbcAMP and PTX. The higher molecular weight protein is unlikely to be related to AKAP 220 however. Although it was interesting that a small population of caudal spermatozoa demonstrated positive staining for AKAP 220 located to the tail region, its presence was not confirmed by Western Blot analysis.

In conclusion, the data indicates that phosphatases involved in dephosphorylation of rat sperm proteins differ in activity, as demonstrated by the differences in the inhibitory effects of Ca^{2+} and Okadaic acid on spermatozoa from different regions of the epididymis. The presence of calcium in the incubation media had a profound inhibitory effect on tyrosine phosphorylation of proteins localised to the tail region, which was particularly evident in caput rat spermatozoa. However, the effect of calcium withdrawal on tyrosine phosphorylation could also be due to a number of other factors such as kinase activation. Consequently, it is crucial that further studies are carried out in order to determine the precise mechanisms by which calcium exerts its inhibitory effects on the capacitation-associated processes discussed in this chapter.

Chapter Eight:

Progesterone inducement of the acrosome reaction in rat epididymal spermatozoa: the effect of alphachlorohydrin

Chapter 8. Progesterone inducement of the acrosome reaction in rat epididymal spermatozoa: the effect of alphachlorohydrin

8.1. Introduction

It is generally accepted that the end point of capacitation is the ability of spermatozoa to undergo the acrosome reaction in response to physiological stimuli such as progesterone and ZP3, a component of the zona pellucida surrounding the oocyte. The acrosome reaction is a regulated secretory event that shares similar characteristics with ligand-receptor-effector activation cascades that have been shown to regulate cellular function in somatic cells. The acrosome itself is a membrane-bound caplike structure covering the anterior portion of the sperm nucleus and although its size and shape varies from species to species, its basic structure is the same in all eutherian mammals (Yanagimachi, 1994) (refer below to Fig. 8.1.). The acrosome is believed to be analogous to a lysosome (Allison and Hartree, 1970) in regards to its enzyme content.

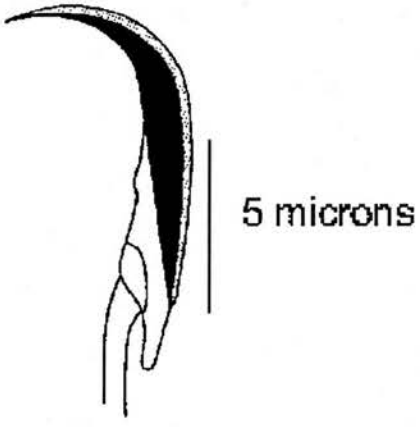


Figure 8.1. Transverse section of a rat sperm head adapted from Yanagimachi (Yanagimachi, 1994). The dotted area represents the acrosome and the black area is the nucleus.

The 'true' acrosome reaction involves multi-fusions between the outer acrosomal membrane and overlying plasma membrane, which enables the hydrolytic enzymic contents of the acrosome escape through the fenestrated membranes (Yanagimachi, 1994). The majority of normal functioning cells will only acrosome react once the process of capacitation has been completed and they are within the vicinity of the oocyte and its investments, where progesterone levels are high. Calcium ionophores, including A23187 (Aitken *et al.*, 1993), progesterone (Melendrez *et al.*, 1994; Murase and Roldan, 1996; Osman *et al.*, 1989; Tesarik *et al.*, 1993a) and zona ligands (Kopf and Gerton, 1990; Wassarman, 1988) have all been employed in the study of the acrosome reaction. However, although the ionophore-induced acrosome reaction is associated with increased phosphoinositide metabolism and activation of phospholipase C (Bennett *et al.*, 1987; Ribbes *et al.*, 1987), it appears to differ structurally from the true physiological acrosome reaction (Watson *et al.*, 1992). It is therefore preferable to employ physiological agonists in the study of the acrosome reaction.

In vivo, mammalian spermatozoa are exposed to progesterone during passage through the cumulus mass (Benoff, 1998) and *in vitro* exposure to progesterone can result in a calcium influx and acrosome reaction (Benoff, 1998). Progesterone was found to induce the acrosome reaction in capacitated stallion spermatozoa (Cheng *et al.*, 1998). The presence of a progesterone receptor on the acrosomal plasma membrane region of the sperm head was indicated and as positive staining for the receptor increased over time, it was postulated that the receptor was gradually unmasked under capacitating conditions (Cheng *et al.*, 1998). Progesterone was also found to induce calcium influx and acrosomal exocytosis in capacitated dog spermatozoa (Brewis *et al.*, 1999). The effects of progesterone on the generation of intracellular messengers such as diacylglycerol (DAG) are mimicked by γ-aminobutyric acid (GABA), suggesting that progesterone acts on a sperm $GABA_A$ receptor. However, it is important to bear in mind that the second messenger pathways activated by progesterone and zona ligands appear to be different (Murase and Roldan, 1996; Tesarik *et al.*, 1993a) and studies indicate that *in vivo*, progesterone by itself, may not induce the acrosome reaction (Carver-Ward *et al.*, 1996; Emiliozzi *et al.*, 1996; Uhler *et al.*, 1992). It is more likely that *in vivo*, progesterone enhances the ability of spermatozoa to undergo a zona pellucida-induced acrosome reaction (Benoff, 1998) and references therein).

As the acrosome reaction is the end point of capacitation of a normal functioning sperm cell, it seemed logical to try and adapt the acrosome reaction test, well established for its use in other species, for use in these studies with rat spermatozoa. If

successful then this assay could provide a useful tool for investigating rat sperm function as well as evaluate possible deleterious effects of various compounds on sperm fertilizing capacity.

The difficulties encountered in obtaining sufficient amounts of ZP3 from native sources and the problems associated with large scale production of glycosylated recombinant ZP3 lead to the choice of progesterone for evaluation as an inducer of the rat sperm acrosome reaction in these studies. Apart from its increased accessibility compared with ZP3, it was also chosen because it is more physiological than the traditionally used calcium ionophores, such as A23187.

The methodology used in this study was adapted from that used by Cooper and Yeung (Cooper and Yeung, 1998). By staining the acrosome with a FITC labelled lectin from peanut agglutinin, it is possible to view under a fluorescence microscope the percentage of cells that have acrosome reacted in comparison with those which have not. As it is important to differentiate between 'true' and 'false' acrosome reactions, the cells were also labelled with ethidium homodimer 1, a vital stain so that live and dead cells may be distinguished. Often dead or degenerative spermatozoa may be autodigested when their membranes lose semipermeability and the outer acrosomal membrane and plasma membrane may be damaged or become detached from the spermatozoon head (Yanagimachi, 1994). Consequently these moribund cells may appear to have undergone the acrosome reaction, when in fact they only exhibited acrosomal loss. In order to distinguish physiological acrosome reactions from such pathological acrosomal loss, it is important to incorporate into acrosome reaction assays a test of cell viability.

The purpose of the studies detailed in this chapter was to establish whether correlations exist between cAMP-mediated increases in tyrosine phosphorylation and the capacity of spermatozoa to undergo the acrosome reaction in response to progesterone treatment. The second aim of this work was to evaluate the suitability of the progesterone-induced acrosome reaction test for reproductive toxicological purposes.

α-Chlorohydrin was chosen because it is a known male reproductive toxicant. However, what distinguishes it from other spermatoxic substances is that although the function of the spermatozoa is severely impaired following exposure to this compound, the cells appear morphologically normal (Jones, 1983). In addition, of particular relevance is the fact that α-chlorohydrin is thought to exert its effect by interfering with various cellular processes such as glycolysis (Jones *et al.*, 1981), which may interfere with capacitation and its associated cellular processes such as tyrosine phosphorylation.

8.2. Materials and Methods

Refer to Chapter 2 for general materials and methods.

8.2.i. The effect of PTX and dbcAMP on the progesterone (P_4)-induced acrosome reaction (AR) in rat epididymal spermatozoa

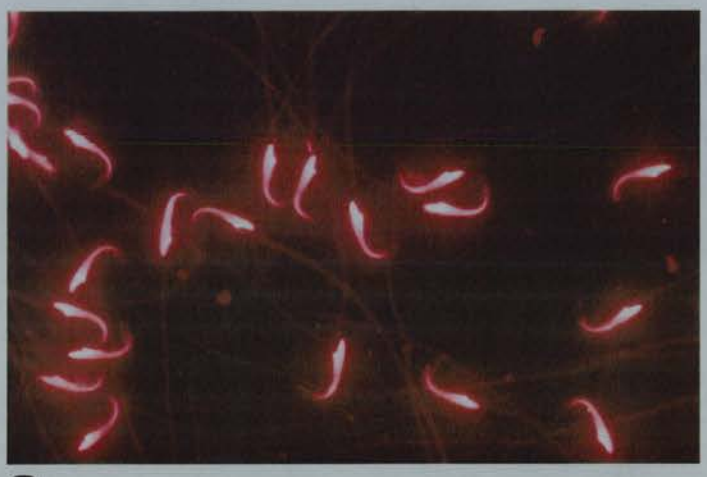
Rat spermatozoa were released into normal BWW as described in Section 2.3., motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. The spermatozoa were incubated with various treatments including 3 mM PTX and 5 mM dbcAMP for 3 hours at 37°C. On completion of the three hour incubation period, P_4 was added to the appropriate sperm treatment at a concentration of 10 μM, for a further 30 minutes at 37°C. An equivalent volume of the BWW/DMSO vehicle excluding the P_4 was added to the other treatments as an internal control. The AR test assay was then performed as detailed in Section 2.18. Refer to Figures 8.2. and 8.3. for photomicrographs of acrosome and vital staining.

In two of the animals, spermatozoa from one epididymis were incubated in Ca^{2+}-free BWW and incubated with various treatments including 3 mM PTX, 5 mM dbcAMP and 2 mM NADPH followed by incubation with 10 μM P_4 as described above. The purpose of this additional experiment was to provide an additional control, as removal of extracellular calcium would be expected to exert an inhibitory effect on the acrosome reaction.

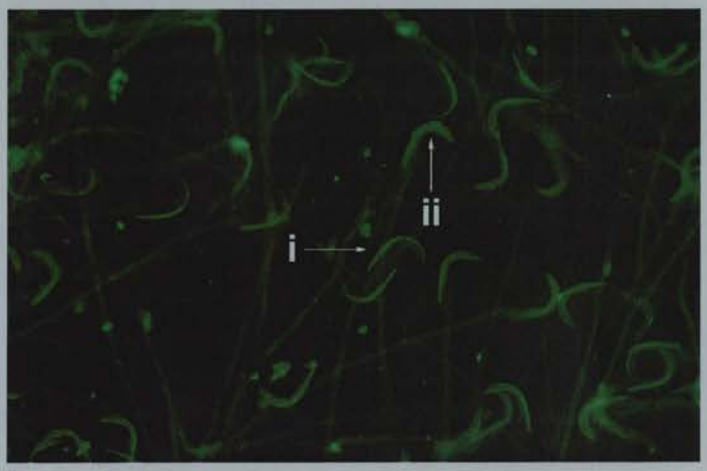
8.2.ii. The effect of PTX and NADPH on the P_4-induced AR in rat epididymal spermatozoa

Rat spermatozoa were released into normal BWW as described in Section 2.3., motility and density counts were performed and the cell concentration adjusted to 10 x 10^6/ml. The spermatozoa were incubated with various treatments including 3 mM PTX and 2 mM NADPH for 3 hours at 37°C. On completion of the three hour incubation period, P_4 was added to the appropriate sperm treatment at a concentration of 10 μM, for a further 30 minutes at 37°C. An equivalent volume of the BWW/DMSO vehicle excluding the P_4 was added to the other treatments as an internal control. The AR test assay was then performed as detailed in Section 2.18. Refer to Figures 8.2. and 8.3. for photomicrographs of acrosome and vital staining.

Figure 8.2. Staining of the acrosome of rat caudal epididymal spermatozoa with lectin from Arachis Hypogea (AH) (FITC) and vital staining with EHD-1 (TRITC). **(a)** This photomicrograph represents spermatozoa that had been frozen at -20°C and then thawed. The bright red fluorescence indicates that these cells are non-viable/dead. **(b)** Photomicrograph representing both live and dead **(i)** acrosome intact and **(ii)** acrosome reacted caudal epididymal spermatozoa. Note how the spermatozoon labelled as (ii), is partially acrosome reacted as there is still a small remnant of fluorescent stained acrosome at the apical region of the head. **(c)** This photomicrograph is identical to (b) and represents the viability of the spermatozoa. By comparing the two images it is possible to differentiate between live cells and dead cells that are acrosome intact or acrosome reacted. Note how the acrosome intact spermatozoa labelled as **(i)** are live as is the acrosome reacted spermatozoon labelled as **(ii)** (magnification x 1000).



a



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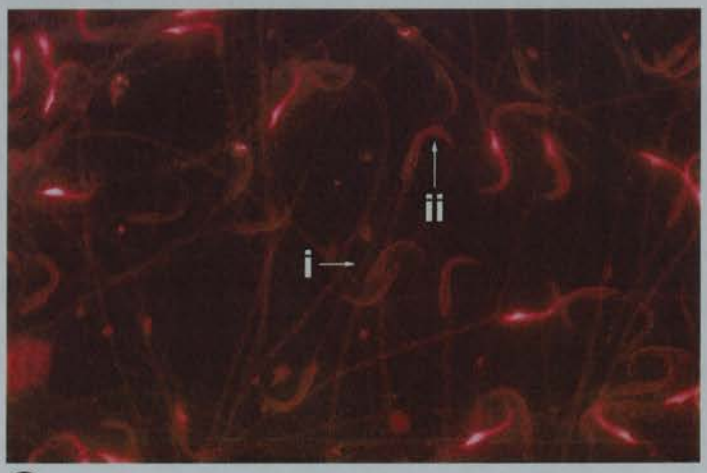
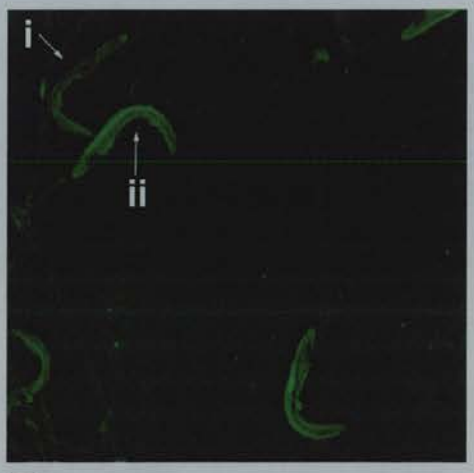
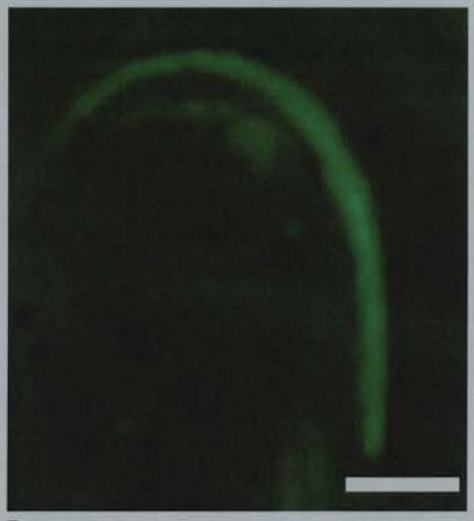


c

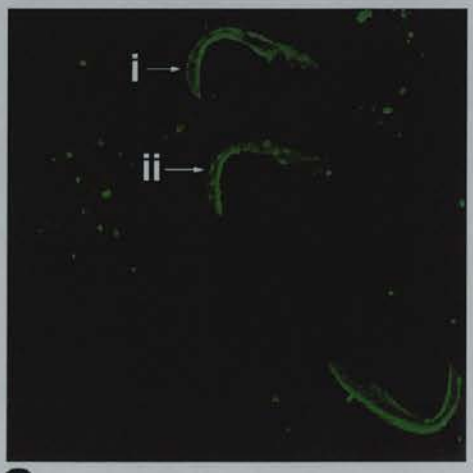
Figure 8.3. Close-up images of the acrosome of caudal spermatozoa stained with AH. **(a) (i)** acrosome reacted spermatozoon, **(ii)** acrosome intact spermatozoon. **(b)** Cropped photomicrograph of an acrosome intact spermatozoon. Note the slight fluorescence on the underside of the head. This region often retains fluorescence even following completion of the acrosome reaction and has been referred to as the acrosomal sac (Oberlander *et al.*, 1996). **(c) (i)** and **(ii)** represent two partially acrosome reacted spermatozoa. Note how they still retain some fluorescence over the posterior region of the acrosome. Scale bar = 4 μm.



a



b



c

8.2.iii. Effect of α-chlorohydrin (α CH) on the P_4-induced AR in rat epididymal spermatozoa *in vitro*

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one of the cauda epididymis was diluted into normal BWW while those from the other were diluted straight into BWW supplemented with 1 mM α CH. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.).

The spermatozoa were incubated with various treatments including 3 mM PTX for 3 hours at 37°C. On completion of the three hour incubation period, P_4 was added to the appropriate sperm treatment at a concentration of 10 μM, for a further 30 minutes at 37°C. An equivalent volume of the BWW/DMSO vehicle excluding the P_4 was added to the other treatments as an internal control. The AR test assay was then performed as detailed in Section 2.18. Refer to Figures 8.2. and 8.3. for photomicrographs of acrosome and vital staining.

8.2.iv. Effect of α-chlorohydrin (α CH) on tyrosine phosphorylation in rat epididymal spermatozoa *in vitro*

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and spermatozoa from one caput epididymis were diluted into normal BWW while those from the other were diluted straight into BWW supplemented with 1 mM α CH. The same procedure was then carried out on the spermatozoa from the caudal region. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.).

The spermatozoa were incubated with various treatments including 3 mM PTX for 3 hours at 37°C. On completion of the three hour incubation period, P_4 was added to the appropriate sperm treatment for a further 30 minutes at 37°C. An equivalent volume of the BWW/DMSO vehicle excluding the P_4 was added to the other treatments as an internal control. Rat sperm proteins were extracted using SDS as described in Section 2.7. and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11.

8.2.v. Effect of α-chlorohydrin (α CH) on the P_4-induced AR in rat epididymal spermatozoa *in vivo*

The *in vivo* work detailed in this chapter was carried out at the Ware site of GlaxoWellcome Research and Development under the study number of R22578, Project licence PPL80/1152 Procedure 1.

The rats used in this study were of the Wistar Han (Charles River Laboratory, UK) variety as this is the chosen strain used in pre-clinical safety studies within GlaxoWellcome Research and Development. Animals used were within an age range of 20 and 24 weeks of age. The animals were maintained within the temperature range of 20-25°C and relative humidity was kept between 45 to 70%. The animals were fed with Rat and Mouse No. 1 Expanded Diet (Special Diets Services Ltd) and water from the domestic supply *ad libitum*.

Animals were orally dosed with various doses (refer to Table 8.1. below) of α CH for five consecutive days and then they were sacrificed on day six by inhalation of a slowly rising concentration of CO_2 followed by dislocation of the cervical region in accordance with Schedule one of the Home Office Animal Act.

Animal Number	Dose of α CH (mg/kg/day)
1-12	0
13-24	1
25-36	3
37-48	9

Table 8.1. The above table represents the doses orally administered to the male rats for five consecutive days. There were twelve animals per dosage group.

Rat spermatozoa were obtained from the epididymis as described in Section 2.3. and diluted in BWW. The spermatozoa were then adjusted to a concentration of 10 x 10^6/ml following density and motility counts (Sections 2.4. and 2.5.).

The spermatozoa were incubated with or without 3 mM PTX for 3 hours at 37°C. On completion of the three hour incubation period, P_4 was added to the appropriate sperm treatment at a concentration of 10 μM, for a further 30 minutes at 37°C. An equivalent volume of the BWW/DMSO vehicle excluding the P_4 was added to the other treatments as an internal control. The AR test assay was then performed as

detailed in Section 2.18. Refer to Figures 8.2. and 8.3. for photomicrographs of acrosome and vital staining.

8.2.vi. Effect of α-chlorohydrin (α CH) on tyrosine phosphorylation in rat epididymal spermatozoa *in vivo*

In addition to the AR test assay, proteins were extracted for Western Blot analysis and slides prepared for immunocytochemistry, from the animals dosed with α CH referred to in Section 8.2.vi. Rat sperm proteins were extracted using SDS as described in Section 2.7. and the proteins separated according to molecular mass by SDS-PAGE (Section 2.9.) and analysed for tyrosine phosphorylation using the Western Blot protocol described in Section 2.11.

In addition, slides were prepared from some of the sperm samples as described in Section 2.14. The cells were fixed with paraformaldehyde and immunocytochemical analysis was carried out on the slides (Section 2.14.) as a way of determining the localisation of tyrosine phosphorylated proteins. Negative controls were set up by incubating the slides with PY20 that had previously been preabsorbed with phospho-L-tyrosine for one hour on a rocker at 37°C. The amount of phospho-L-tyrosine was calculated by incubating 10 x the amount of protein in the PY20 antibody, e.g. 0.350 mg of PY20 was incubated with 3.5 mg of phospho-L-tyrosine.

8.3. Results

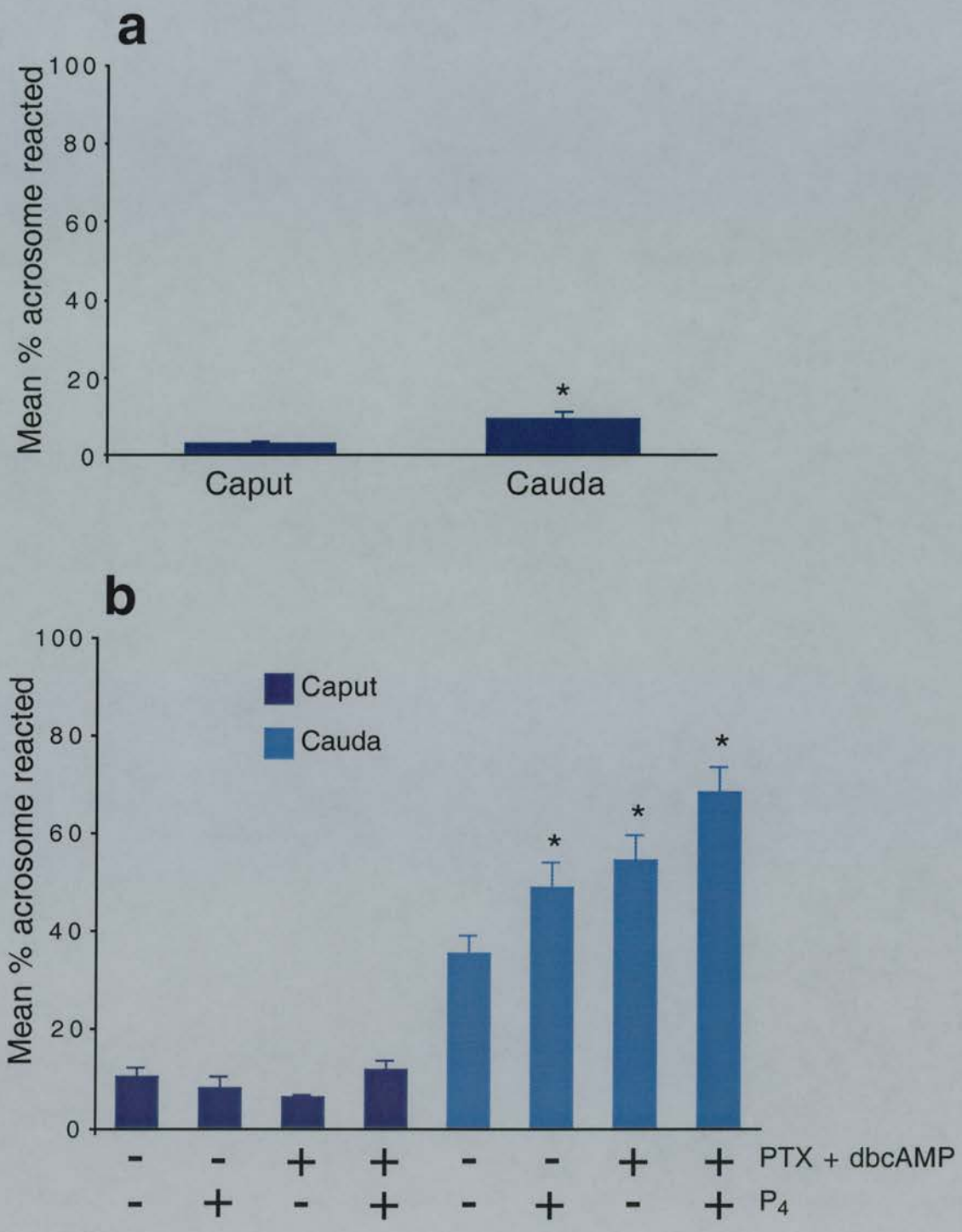
8.3.i. The effect of PTX and dbcAMP on the progesterone (P_4)-induced acrosome reaction (AR) in rat epididymal spermatozoa

In addition to spermatozoa incubated with PTX and dbcAMP, the acrosome reaction status of untreated caput and caudal spermatozoa at the beginning of the incubation period was also evaluated. The results obtained indicated that spontaneous ARs in both caput and caudal rat spermatozoa were relatively low, less than 10% average for spermatozoa irrespective of their level of epididymal maturation (Fig. 8.4a. and Table 8.2.). However, caudal spermatozoa had significantly higher numbers of spontaneous ARs in comparison with those obtained from the caput region (Fig. 8.4b. and Table 8.2.).

Following three and a half hours incubation with various treatments, caput spermatozoa demonstrated increased levels of ARs in comparison with the number of AR's at the beginning of the incubation. However, levels still remained less than a mean of 15% and there was no significant difference between treatments (Fig. 8.4. and Table 8.2.). In contrast, caudal spermatozoa demonstrated a significantly increased population of acrosome reacted cells, when compared with the average population at the beginning of the incubation (Fig. 8.4. and Table 8.2.). In addition all caudal spermatozoa, irrespective of the treatment, demonstrated significantly higher levels of ARs than the caput spermatozoa (Fig. 8.4b. and Table 8.2.). All treated caudal spermatozoa had significantly higher levels of ARs than the untreated control caudal spermatozoa. Caudal spermatozoa that had been incubated with PTX, dbcAMP and P_4, consisted of a significantly greater population of acrosome reacted cells than all other treatments (Fig. 8.4b. and Table 8.2b.).

Spermatozoa that had been incubated with the above treatments in Ca^{2+}-free BWW demonstrated very low levels of acrosome reactions. Spermatozoa incubated for 3 hours with 3 mM PTX and 5 mM dbcAMP followed by a further 30 minutes with 10 μM P_4 demonstrated a maximum of 11% acrosome reactions (n = 2).

Figure 8.4. The effect of PTX and dbcAMP on the P_4-induced acrosome reaction in rat epididymal spermatozoa. Percentage population of live acrosome reacted rat epididymal spermatozoa, at **(a)** the beginning of the incubation and **(b)** following incubation with 3 mM PTX and 5 mM dbcAMP for three hours followed by 10 μM P_4 for a further 30 minute incubation at 37°C (n = 4).

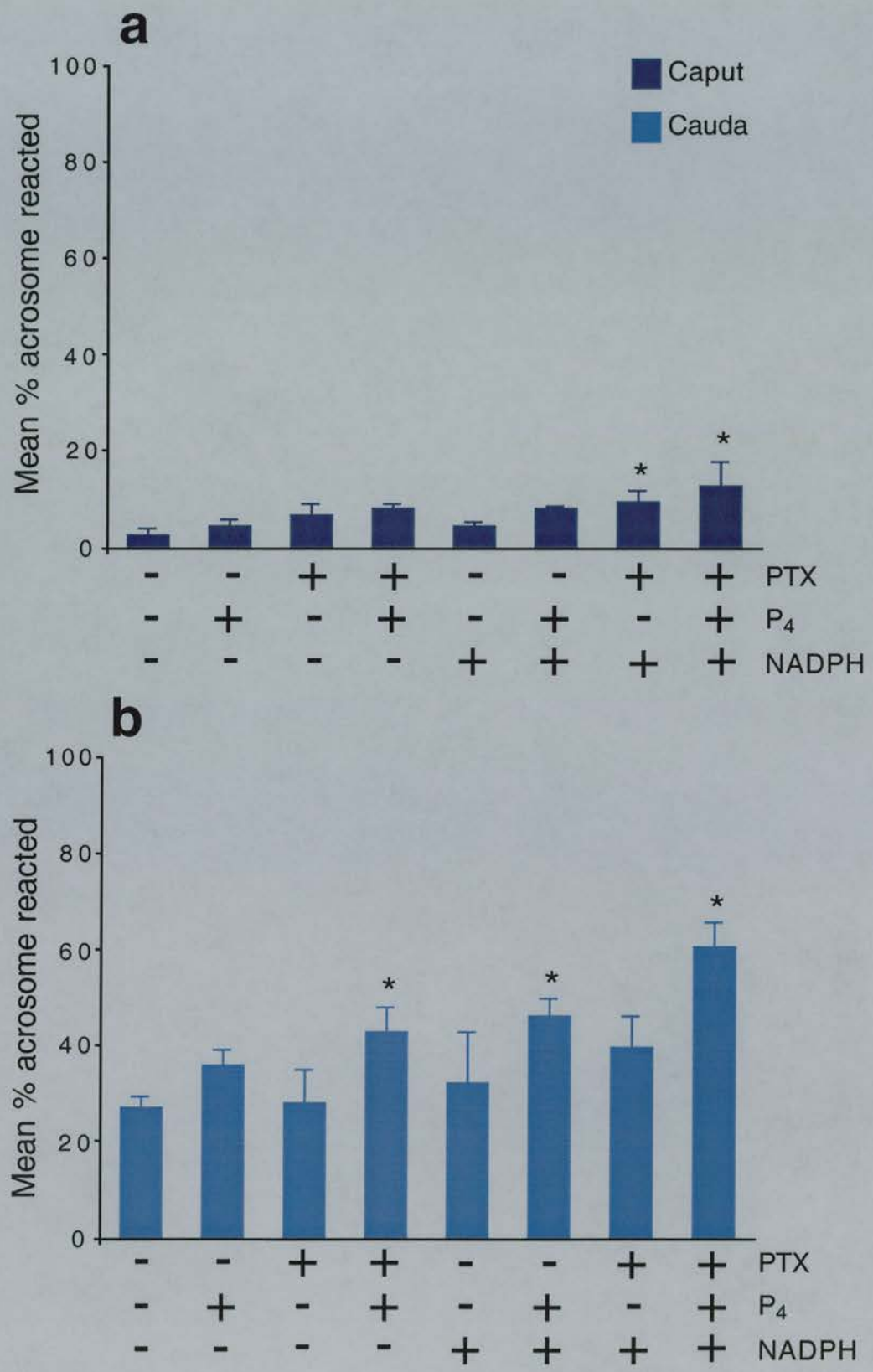

a
Mean % acrosome reacted
100
80
60
40
20
0
*
Caput
Cauda
b
Mean % acrosome reacted
100
80
60
40
20
0
Caput
Cauda
*
*
*
- - + + - - + + PTX + dbcAMP
- + - + - + - + P4

8.3.ii. The effect of PTX and NADPH on the P_4-induced AR in rat epididymal spermatozoa

All caput spermatozoa consisted of relatively low levels of acrosome reacted cells, with mean percentages per population less than 15% for all of the treatments (Fig. 8.5a. and Table 8.2c.). All caudal spermatozoa exhibited significantly increased numbers of AR's in comparison with caput spermatozoa (Fig. 8.5. and Table 8.2c.). Interestingly, only caudal spermatozoa that had been treated with P_4, had significantly greater numbers of acrosome reacted cells than the control untreated caudal spermatozoa (Fig. 8.5b. and Table 8.2c.). Those caudal spermatozoa incubated with a combination of PTX, NADPH and P_4 had significantly more AR's than those incubated with either P_4 alone, PTX and NADPH, NADPH alone or PTX with P_4 (Fig. 8.5b. and Table 8.2c.).

Spermatozoa that had been incubated with the above treatments in Ca^{2+}-free BWW demonstrated very low levels of acrosome reactions. Spermatozoa incubated for 3 hours with 3 mM PTX and 2 mM NADPH followed by a further 30 minutes with 10 μM P_4 demonstrated a maximum of 9% acrosome reactions (n = 2).

Figure 8.5. The effect of PTX and NADPH on the P_4-induced acrosome reaction in rat epididymal spermatozoa. Percentage population of live acrosome reacted **(a)** caput and **(b)** caudal rat epididymal spermatozoa following incubation with 3 mM PTX and 2 mM NADPH for three hours followed by 10 μM P_4 for a further 30 minute incubation at 37°C (n = 3).


a
Caput
Cauda
Mean % acrosome reacted
100
80
60
40
20
0
*
*
- - + + - - + + PTX
- + - + - + - + P_4
- - - - + + + + NADPH
b
Mean % acrosome reacted
100
80
60
40
20
0
*
*
*
- - + + - - + + PTX
- + - + - + - + P_4
- - - - + + + + NADPH

Spermatozoa	Mean % AR	Standard error
Caput	3	0.816
Cauda	9.25	2.016

a

Treatments	Caput		Cauda	
	Mean % AR	Standard error	Mean % AR	Standard error
Control	10.625	1.886	35.5	3.753
P_4	8.25	2.287	48.75	5.121
PTX+dbcAMP	6.5	0.5	54.5	5.058
PTX+dbcAMP +P_4	12	1.581	68.5	5.008

b

Treatment	Caput		Cauda	
	Mean % AR	Standard error	Mean % AR	Standard error
Control	2.667	1.202	27	2.517
P_4	4.333	1.453	36	3.055
PTX	7	2.082	28	7.234
PTX+P_4	8.333	0.667	42.667	5.364
NADPH	4.333	0.882	32.333	10.477
NADPH+P_4	8	0.577	46	4.041
PTX+NADPH	9.333	2.603	39.667	6.36
PTX+NADPH +P_4	13	4.933	60.333	5.364

c

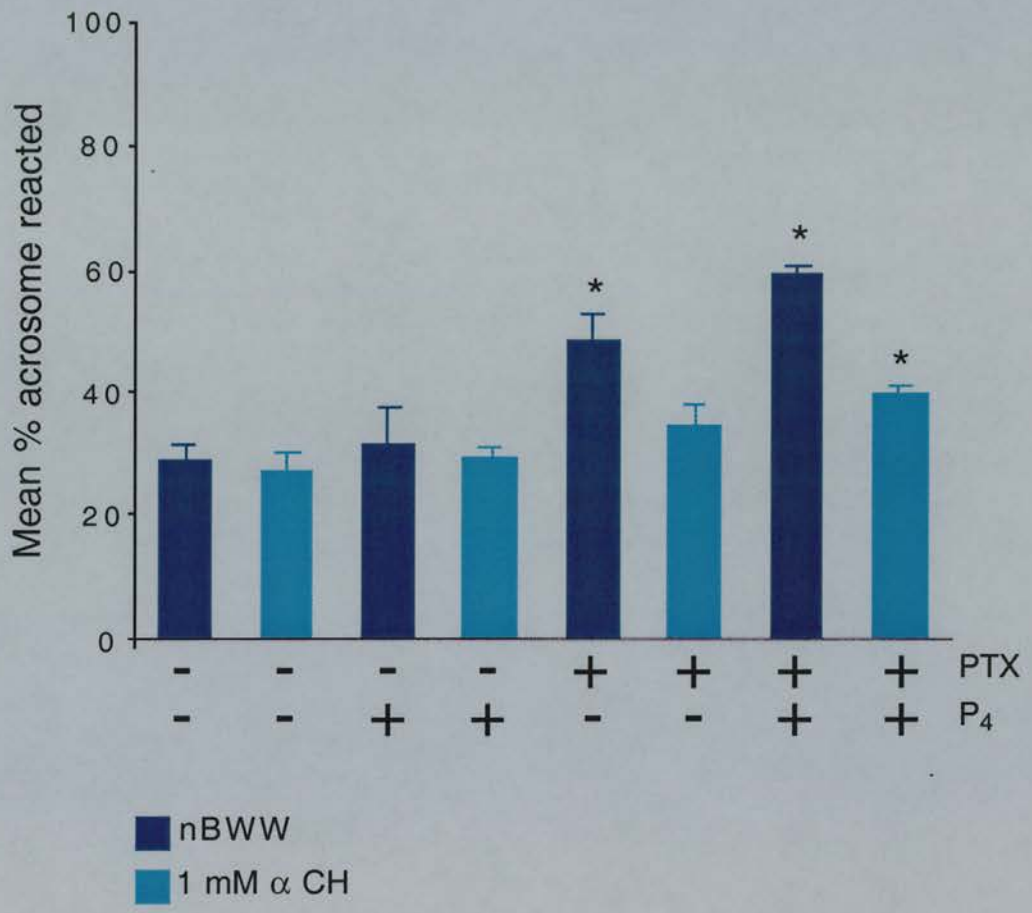
Table 8.2. The tables above represent the mean percentage population of live acrosome reacted rat caudal spermatozoa following incubation with various treatments for up to 3.5 hours at 37°C. **(a)** n = 4 **(b)** n = 4 **(c)** n = 3

8.3.iii. Effect of α-chlorohydrin (α CH) on the P_4-induced AR in rat epididymal spermatozoa *in vitro*

As caput spermatozoa did not appear to respond to any treatments in regards to induction of the acrosome reaction (refer to Sections 8.2.i. and 8.2.ii.), they were not included in the following analysis of acrosome reaction status. Consequently the data presented is representative of spermatozoa obtained from the cauda epididymis only.

Spermatozoa incubated with either PTX alone or PTX in combination with P_4 consisted of significantly greater populations of acrosome reacted spermatozoa than the untreated control sperm cells (Fig.8.6. and Table 8.3.). Spermatozoa treated with both PTX and P_4 demonstrated significantly more AR's than all other treatments (Fig.8.6. and Table 8.3.). α-Chlorohydrin did not exert a significant effect on the percentage population of acrosome reacted spermatozoa, except in those spermatozoa that had been treated with PTX in combination with P_4 (Fig.8.6. and Table 8.3.). However, AR's were considerably reduced when compared with the same treatment in normal BWW (Fig.8.6. and Table 8.3.).

Figure 8.6. The effect of α CH on the acrosome reaction in rat caudal epididymal spermatozoa. Percentage population of live acrosome reacted caudal rat epididymal spermatozoa, following incubation in either nBWW or BWW supplemented with 1 mM α CH, with 3 mM PTX for three hours, followed by 10 μM P_4 for a further 30 minute incubation at 37°C (n = 4).


Mean % acrosome reacted
100
80
60
40
20
0
*
*
*
- - - - + + + + PTX
- - + + - - + + P4
nBWW
1 mM α CH

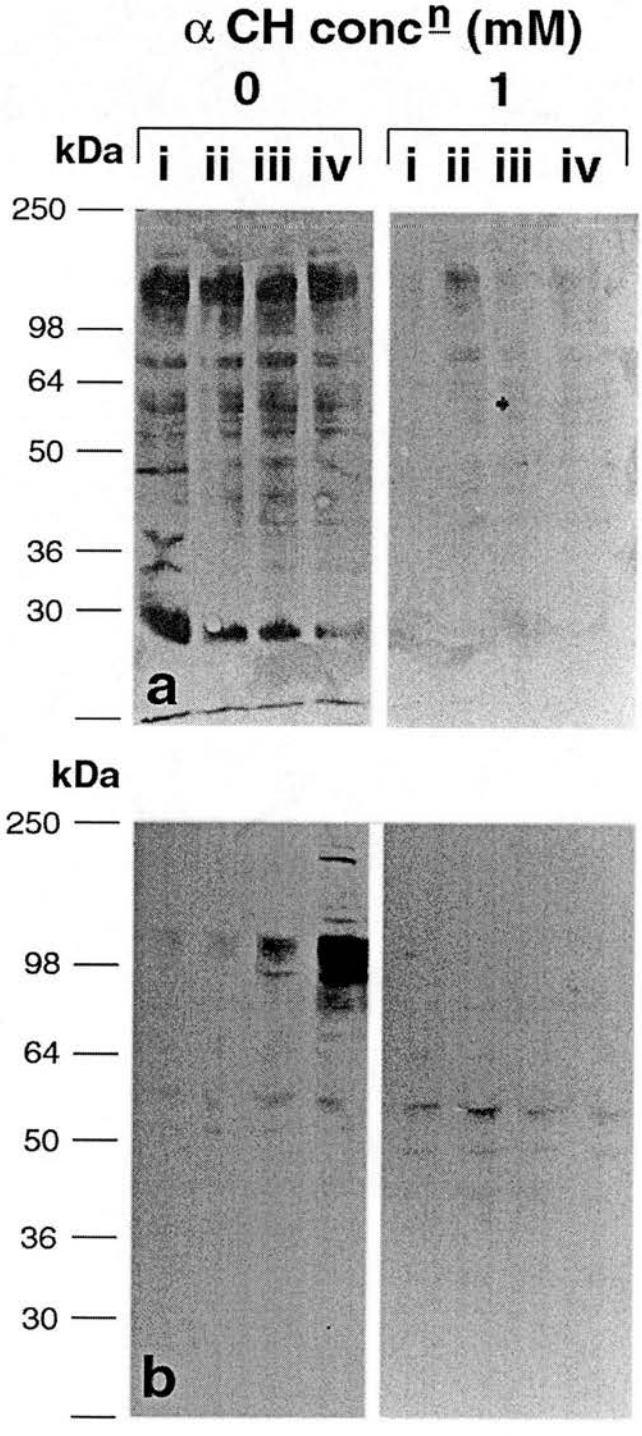
Treatment	Mean % AR	Standard error
Control	28.5	3.279
P_4	31.25	5.879
PTX	48.25	4.131
PTX+P_4	58.75	1.601
α CH/control	27	3.028
α CH/P_4	29.25	1.315
α CH/PTX	34.5	3.403
α CH/ PTX+P_4	39.25	1.436

Table 8.3. The above table represents the mean percentage population of live acrosome reacted rat caudal spermatozoa following incubation with various treatments for 3.5 hours at 37°C (n = 4).

8.3.iv. Effect of α-chlorohydrin (α CH) on tyrosine phosphorylation in rat epididymal spermatozoa *in vitro*

None of the treatments appeared to have any effect on tyrosine phosphorylation in caput epididymal spermatozoa (Fig. 8.7a.). However, exposure to α CH almost completely inhibited tyrosine phosphorylation, irrespective of the treatment (Fig. 8.7a.). In contrast, when caudal spermatozoa were treated with the combined treatment of PTX and P_4, tyrosine phosphorylation was actually up-regulated in comparison with the controls and the singular treatments (Fig. 8.7b.). Phosphorylation was induced in proteins of approximately 220, 200 and 130 kDa, although the intensity was relatively low (Fig. 8.7b.), while intense phosphorylation was observed in proteins of approximately 76-110, 130 and 185 kDa (Fig. 8.7b.). Alternatively, caudal spermatozoa that had been exposed to α CH exhibited almost complete down-regulation of tyrosine phosphorylation except for proteins of approximately 50 and 56 kDa.

Figure 8.7. The effect of α CH on tyrosine phosphorylation in rat epididymal spermatozoa. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from **(a)** caput and **(b)** caudal spermatozoa with 1% SDS. The spermatozoa had previously been incubated in either nBWW or BWW supplemented with 1 mM α CH, with 3 mM PTX for three hours, followed by 10 μM P_4 for a further 30 minute incubation at 37°C. **(i)** control, **(ii)** 10 μM P_4, **(iii)** 3 mM PTX, **(iv)** 3 mM PTX + 10 μM P_4 (n = 5).

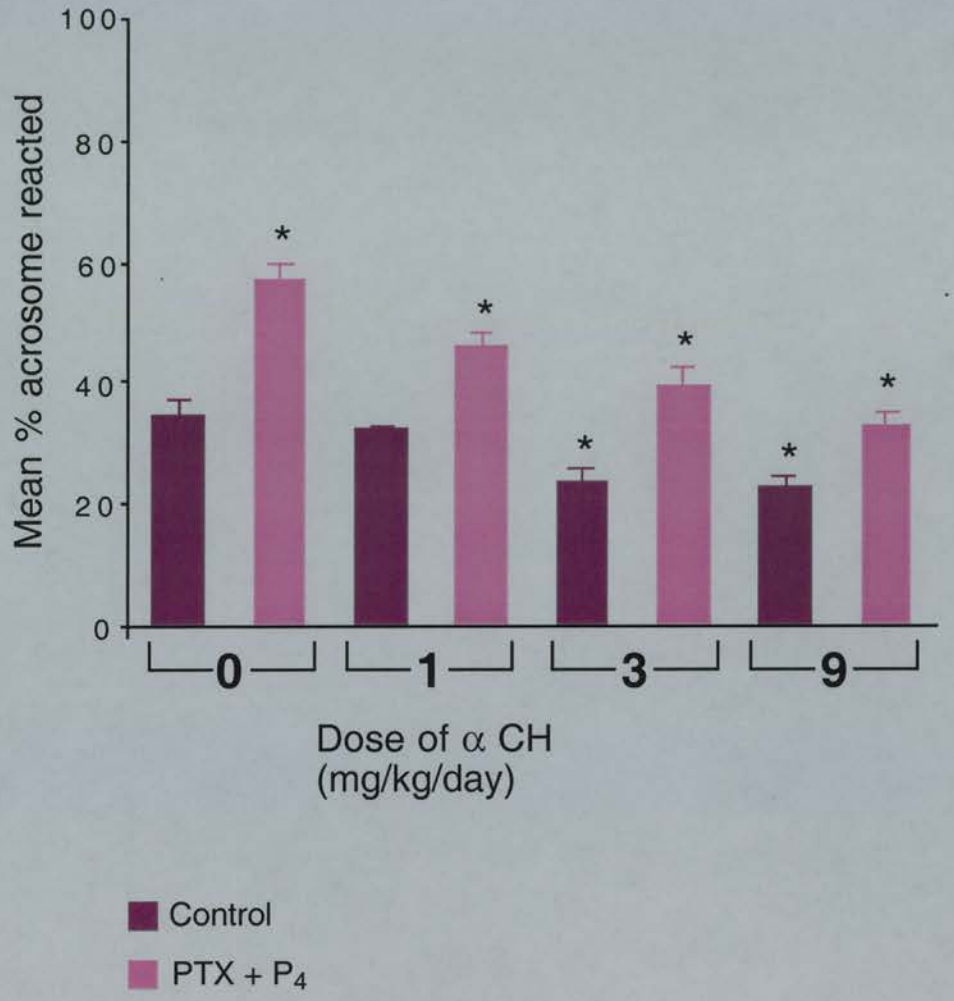

α CH conc^{n} (mM)
0
1
kDa
i ii iii iv
i ii iii iv
250
98
64
50
36
30
a
kDa
250
98
64
50
36
30
b

8.3.v. Effect of α-chlorohydrin (α CH) on the P_4-induced AR in rat epididymal spermatozoa *in vivo*

As caput spermatozoa did not appear to respond to any treatments in regards to induction of the acrosome reaction (refer to Sections 8.2.i. and 8.2.ii.), they were not included in the following analysis of acrosome reaction status.

Levels of spontaneous ARs were significantly higher in spermatozoa that had not been exposed to α CH than those that had been exposed to 3 and 9 mg/kg/day (Fig. 8.8. and Table 8.4.). All spermatozoa that had been treated with PTX and P_4 consisted of significantly greater populations of acrosome reacted spermatozoa than their corresponding untreated samples, irrespective of α CH dosage (Fig. 8.8. and Table 8.4.). In addition PTX and P_4 stimulated spermatozoa that had been exposed to any level of α CH exhibited significantly reduced numbers of ARs in comparison with those treated cells that had not been exposed to α CH (Fig. 8.8. and Table 8.4.). PTX and P_4 stimulated spermatozoa, obtained from rats that had been dosed with 9 mg/kg/day α CH had significantly lower levels of acrosome reacted cells than those exposed to the minimum dose, although there was no significant difference in ARs between those at the higher dose and the spermatozoa exposed to 3 mg/kg/day (Fig. 8.8. and Table 8.4.).

Figure 8.8. The effect of α CH on the acrosome reaction in rat caudal epididymal spermatozoa. Percentage population of live acrosome reacted caudal rat epididymal spermatozoa, extracted from rats that had been orally administered various doses of α CH for five consecutive days. The animals were sacrificed on the sixth day and the spermatozoa extracted. The caudal spermatozoa were incubated with 3 mM PTX for three hours, followed by 10 μM P_4 for a further 30 minute incubation at 37°C (n = 12). The control treatments are representative of the spontaneous AR rate following 3.5 hours incubation.


Mean % acrosome reacted
100
80
60
40
20
0
*
*
*
*
*
*
0
1
3
9
Dose of α CH
(mg/kg/day)
Control
PTX + P4

	Control		PTX+P_4	
Dosage of α CH (mg/kg/day)	**Mean % AR**	**Standard error**	**Mean %AR**	**Standard error**
0	34.636	2.545	57.136	2.6
1	32.167	1.061	46.208	1.864
3	23.667	1.878	39.25	3.215
9	23.042	1.507	33.1	1.949

Table 8.4. The above table represents the mean percentage population of live acrosome reacted rat caudal spermatozoa following incubation with various treatments for 3.5 hours at 37°C. The animals had been orally administered with various doses of α CH for five consecutive days, prior to sacrifice and extraction of the spermatozoa for analysis on day six (n = 12 animals/treatment group).

8.3.vi. Effect of α-chlorohydrin (α CH) on tyrosine phosphorylation in rat epididymal spermatozoa *in vivo*

The effect of *in vivo* administration of α CH on tyrosine phosphorylation did not appear to be as substantial when compared to incubating the spermatozoa directly with the compound *in vitro* (Fig. 8.7. and 8.9). There did appear to be some inhibition of this process that was clearly dose dependent, particularly in the low molecular weight proteins below approximately 86 kDa in caput spermatozoa and above approximately 86 kDa in caudal spermatozoa (Fig. 8.9.). As predicted, α CH only appeared to severely affect tyrosine phosphorylation at the maximum dosage of 9 mg/kg/day, a dosage that has been demonstrated to completely inhibit fertilisation (work carried out at GlaxoWellcome as part of Joanne Woods MSc thesis). However, the impact of such a dosage on this process was still not equal to that exerted by the *in vitro* exposure to α CH (Fig. 8.7. and 8.9.). There was also a lot of variability between

animals as well as protein degradation in the extracts and consequently the quality of the Western Blots was disappointingly inadequate in many of the samples.

Immunocytochemistry demonstrated that the percentage population exhibiting tyrosine phosphorylation of the entire acrosomal domain was significantly reduced in caput spermatozoa that had been exposed to levels of 3 and 9 mg/kg/day, irrespective of the treatment, when compared with those that had not (Fig. 8.10a.). *In vivo*, α CH exposure did not appear to significantly decrease levels of tyrosine phosphorylation in the posterior margin of the acrosome in caudal spermatozoa (Fig. 8.10b.).

Figure 8.9. The effect of α CH on tyrosine phosphorylation in rat epididymal spermatozoa. A Western Blot of tyrosine phosphorylated proteins probed with PY20 following extraction from **(a)** caput and **(b)** caudal spermatozoa with 1% SDS. The spermatozoa were extracted from rats that had been orally administered various doses of α CH for five consecutive days. The animals were sacrificed on the sixth day and the spermatozoa extracted. The spermatozoa were incubated with 3 mM PTX for three hours, followed by 10 μM P_4 for a further 30 minute incubation at 37°C. **(i)** Control, **(ii)** 3 mM PTX + 10 μM P_4 (n = 6).

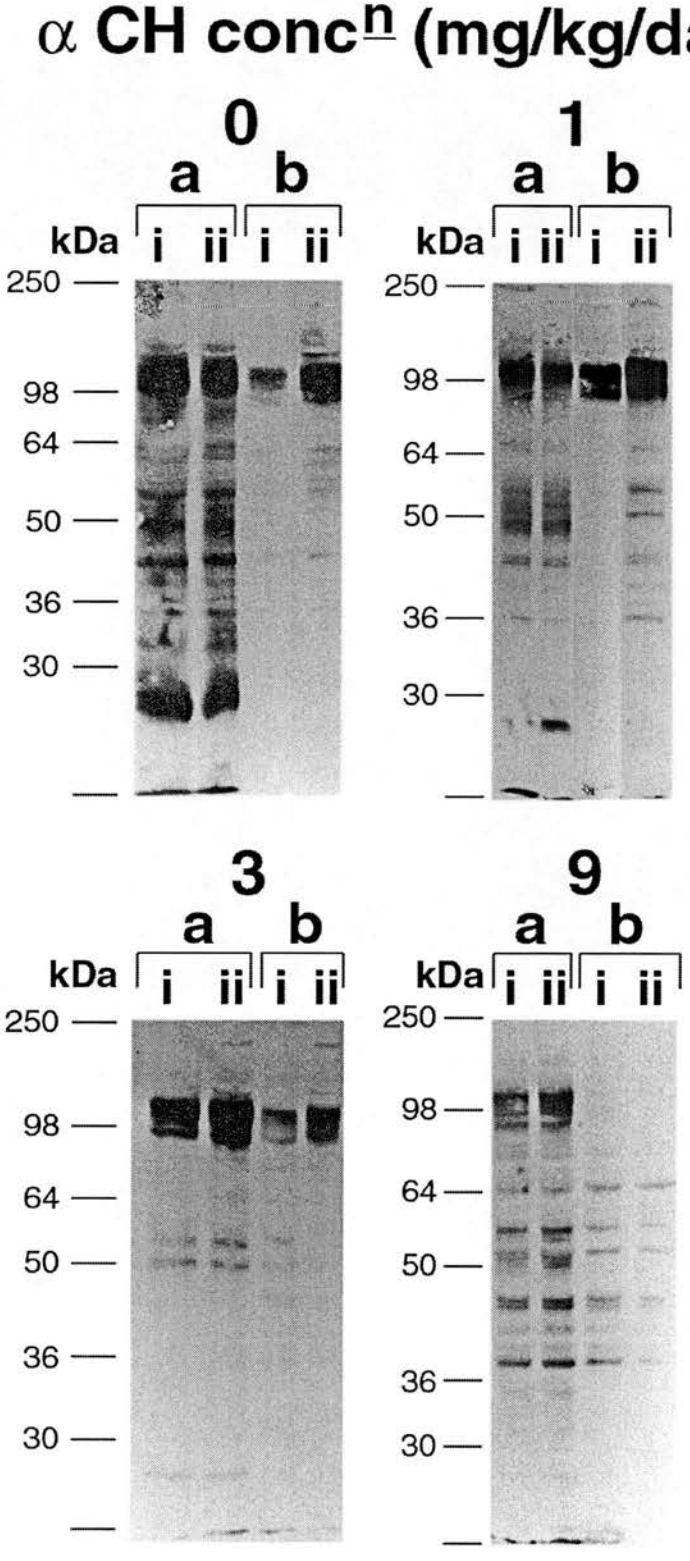
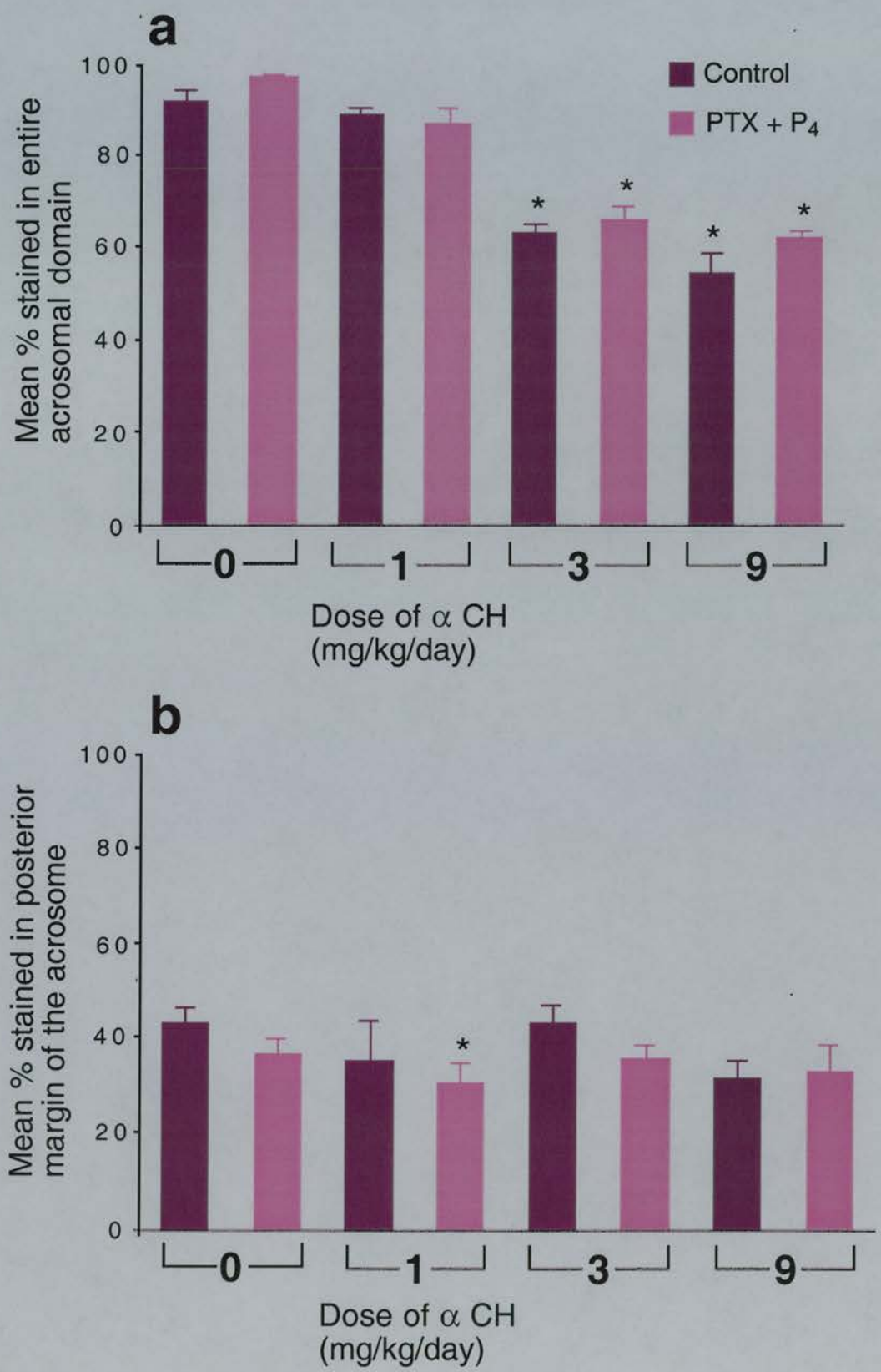

α CH conc^n (mg/kg/day)
0
1
3
9
a
b
i
ii
kDa
250
98
64
50
36
30

Figure 8.10. The effect of α CH on tyrosine phosphorylation in rat epididymal spermatozoa. Immunolocalisation of tyrosine phosphorylated proteins in **(a)** caput and **(b)** caudal spermatozoa. The spermatozoa were extracted from rats that had been orally administered various doses of α CH for five consecutive days. The animals were sacrificed on the sixth day and the spermatozoa extracted. The spermatozoa were incubated with 3 mM PTX for three hours, followed by 10 μM P_4 for a further 30 minute incubation at 37°C (n = 3).


a
Control
PTX + P4
Mean % stained in entire acrosomal domain
100
80
60
40
20
0
*
*
*
*
0
1
3
9
Dose of α CH
(mg/kg/day)
b
Mean % stained in posterior margin of the acrosome
100
80
60
40
20
0
*
0
1
3
9
Dose of α CH
(mg/kg/day)

8.4. Discussion

The data represented here clearly indicate fundamental differences in the ability of rat spermatozoa to undergo the acrosome reaction that is evidently related to their epididymal maturation status. Both caput and caudal spermatozoa demonstrated relatively low levels of ARs when analysed immediately following release into BWW, although numbers were significantly higher in the caudal sperm cells. However, following three and a half hours incubation in BWW, the spontaneous acrosome rate significantly increased to relatively high levels in caudal spermatozoa, when compared to what has been observed for other species. The average spontaneous acrosome reaction rate of spermatozoa in the mouse has been demonstrated to be around 22% (Murase and Roldan, 1996), 19.5% in the Asiatic elephant (Kitiyanant *et al.*, 2000) and 9% in humans (de Jonge *et al.*, 1991). This higher level of acrosome reactions was consistent throughout all of the studies. However, spontaneous ARs in caput sperm remained low and this was irrespective of any of the treatments.

There are several possible explanations for the relatively high percentage population of spontaneous acrosome reacted cells in caudal spermatozoa. It could be due to the fact that the membranes and cytoskeleton of caput cells are less pliable than the caudal spermatozoa, thus leading to increased susceptibility of the mature cells losing their acrosomes, through the spinning and washing procedures required for the assay. Clearly a powerful factor determining AR rates is the fact that only capacitated spermatozoa can acrosome react in response to progesterone and caput epididymal spermatozoa are incapable of capacitation. Indeed, the basal level of AR observed in the caput cells gives a good approximation of the spurious AR rate generated in the system as a result of the detection method. However, the fact that dosing rats with α CH lead to a concentration dependent decrease in spontaneous acrosome reactions, suggests that such high levels of spontaneous acrosome reactions cannot simply be due to the cell processing procedures, as all spermatozoa underwent the same centrifugation protocol.

Although the acrosome reaction was not induced in caput spermatozoa, following incubation with various cAMP stimulants, observations in caudal spermatozoa were very different. Untreated caudal spermatozoa, exposed to P_4 exhibited significantly increased AR's, in comparison with the spontaneous ARs observed in the untreated control cells. This suggests that a population of caudal spermatozoa are capable of undergoing capacitation during incubation in BWW, leading to their increased susceptibility to the presence of P_4, in regard to its inducement of the acrosome reaction. Treatment of caudal spermatozoa with PTX and dbcAMP, followed by

exposure to P_4, lead to a significantly greater population of acrosome reacted cells than in any of the other treatments. Interestingly, this correlated with levels of tyrosine phosphorylation, as maximum levels were achieved by increasing intracellular cAMP levels through the combination of PTX and dbcAMP. A rational interpretation of these data is that cAMP promotes capacitation through the induction of tyrosine phosphorylation and that progesterone is only capable of inducing the acrosome reaction in capacitated cells.

Intracellular levels of cAMP are elevated during the acrosome reaction (Hyne and Garbers, 1979a; Ward and Kopf, 1993). It has been postulated that the role of cAMP in the acrosome reaction may be to facilitate the release of calcium from an acrosomal store, which is partially inhibited by the PKA inhibitor, H89 (Spungin and Breitbart, 1996). This raises the possibility of the presence on the acrosomal membrane of a cAMP-gated calcium channel, or a channel opened upon phosphorylation by PKA (Breitbart and Spungin, 1997). A calcium channel opened by cAMP has been detected in sea urchin spermatozoa (Cook and Babcock, 1993). More recently, cAMP has been shown to stimulate calcium-dependent exocytosis of the sperm acrosome, acting downstream of phospholipase A2 (Garde and Roldan, 2000). The presence of a cAMP-gated calcium channel in rat spermatozoa would explain why the treatment of caudal spermatozoa with PTX and cAMP significantly raised the proportion of acrosome reacted spermatozoa even without subsequent exposure to P_4.

The acrosome reaction failed to be induced in caput spermatozoa that had been incubated with a combination of treatments including PTX and NADPH, followed by exposure to P_4. In contrast, both PTX and NADPH appeared to increase the proportion of acrosome reacted caudal spermatozoa, when incorporated into the incubation media, either singularly, or in combination. However, such treatments only significantly raised levels of AR's, when compared to the untreated controls, if they were also exposed to P_4. This indicates that although NADPH and PTX prime the cells to undergo the acrosome reaction, there is a requirement for P_4 for the completion of the process.

It is unclear why caput spermatozoa possess such a limited ability to undergo the acrosome reaction. However, as demonstrated with the process of tyrosine phosphorylation, there are mechanisms in place for the inhibition of such maturational processes. Caudal rat spermatozoa analysed for their capacitation and acrosomal status using the chlortetracycline assay, demonstrated that an intermediate pattern representing the capacitated, but acrosome intact state, consisted of a dark band in the postacrosomal region of the sperm head (Oberlander *et al.*, 1996). It was also demonstrated in mouse spermatozoa that this dark band represented the capacitated

state (Saling and Storey, 1979; Ward and Storey, 1984). This dark band is the exact region where caudal spermatozoa exhibit tyrosine phosphorylated proteins. This raises the intriguing possibility that the restriction of phosphorylation from the entire acrosomal domain in immature caput spermatozoa, to a discreet band in the posterior margin of the acrosome of caudal cells, is an important maturational event for the acquisition of the capacitated state.

Other important maturation-dependent changes that have been demonstrated include biochemical alterations in the proacrosin-acrosin system, during epididymal maturation of rat spermatozoa (Nagdas *et al.*, 1992). These changes involve the maturation-dependent appearance of several low molecular weight forms of proacrosin-acrosin (Nagdas *et al.*, 1992). During epididymal transit, the 52 kDa precursor form, proacrosin is partially converted to the low molecular weight components of 41, 34 and 31 KDa (Nagdas *et al.*, 1992). These studies indicate that a sperm cell must undergo important intra-acrosomal events during epididymal transit in order to become a functionally mature sperm cell (Nagdas *et al.*, 1992). *In vivo*, it is very important that the acrosome reaction does not take place prematurely, as this would prevent fertilisation, due to the inability of the spermatozoa to penetrate the oocyte investments. As the acrosome contents are an absolute requirement for the penetration of the cumulus oophorus and the zona pellucida, timing of the acrosome reaction is crucial.

For the second part of the study, the aim was to observe the effects of a known reproductive toxicant on the process of tyrosine phosphorylation and the acrosome reaction in rat epididymal spermatozoa. Due to the fact that caput spermatozoa do not appear to undergo the acrosome reaction in response to various stimulants, they were not analysed for their acrosomal status as part of this study.

α CH was chosen as it has been proven to be a potent male reproductive toxicant (Jones, 1978; Jones, 1983). It acts at the epididymis and adversely affects sperm energy metabolism (Cooney and Jones, 1988; Ford and Harrison, 1987). It has no obvious effect on sperm morphology, and testicular and epididymal pathology, are only observed in rats that have received doses above 20 mg/kg/day, which exceeds the dose required for complete inhibition of fertilisation (Woods, 1994).

α CH is thought to exert its effect on spermatozoa by interfering with the glycolytic pathway. A metabolite of α CH, (S)-3-chlorolactaldehyde, has been proposed as an inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase (3-GPD) (Jones *et al.*, 1981; Stevenson and Jones, 1981). This enzyme is an important component of the glycolytic pathway and inhibition leads to inhibition of the sperm cell's ability to generate ATP, by both oxidative and substrate level phosphorylation (Fig.8.11.). A second proposal was put forward by Ford and Harrison (Ford and

Harrison, 1987) suggesting that inhibition of 3-GPD led to the accumulation of another intermediate in the glycolytic pathway, fructose 1,6-bisphosphate (Fig.8.11.). A build up of this product pushes the glycolytic pathway in the other direction, contributing to ATP dissipation by initiating futile substrate cycling (Fig.8.11.).

In vitro work, carried out by Joanne Woods (Woods, 1994), as part of her MSc thesis, indicated that α CH inhibited fertilisation, even when the sperm were placed close within the vicinity of the ova for IVF purposes. This could be due to compromise of the sperm's ability to achieve the capacitated state and its associated hyperactivated motility, behaviour important for the penetration of the oocyte and its investments.

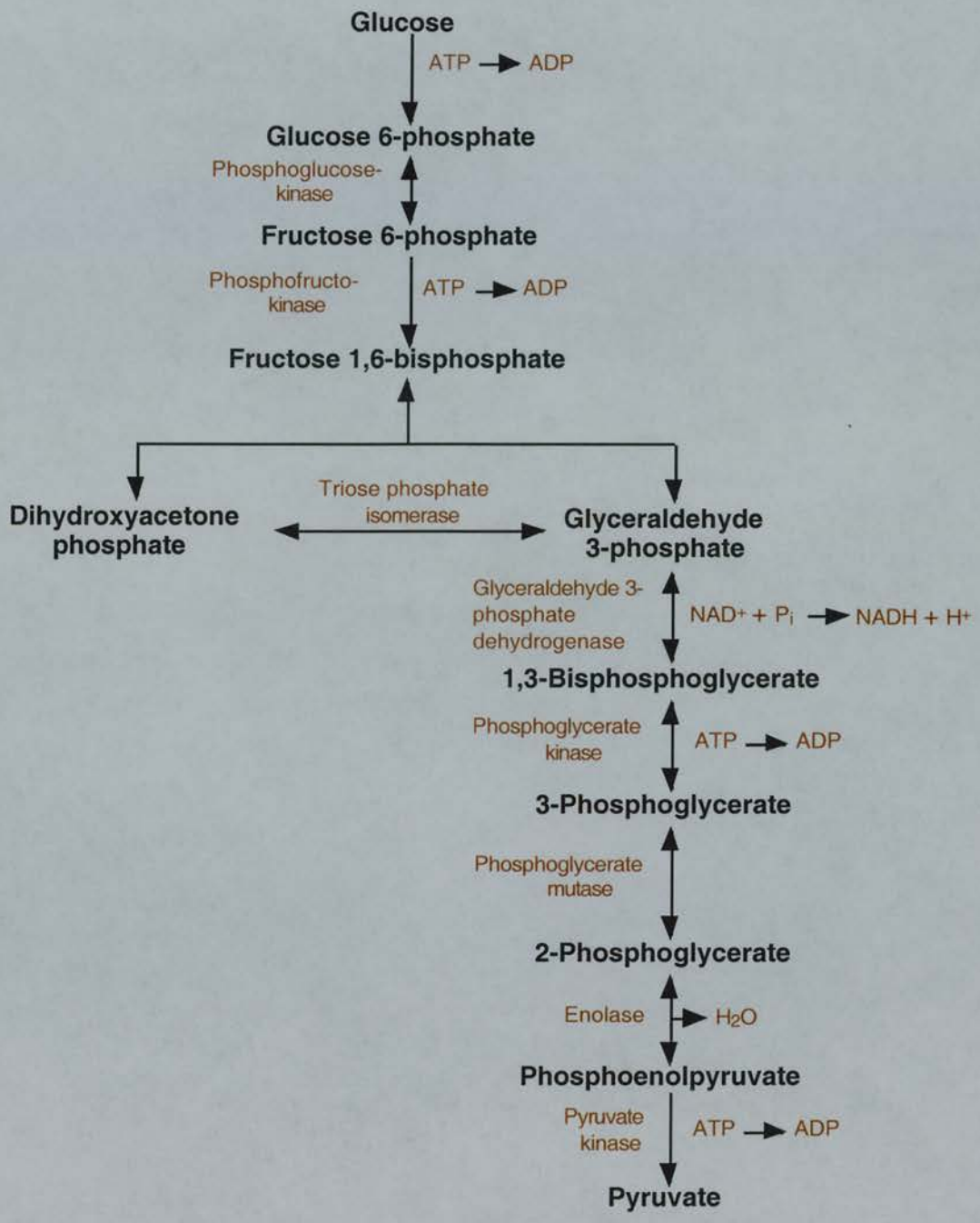


Figure 8.11. The glycolytic pathway. Adapted from (Stryer, 1988).

If α CH exerts its inhibitory effect on sperm by interfering with the glycolytic pathway and ATP levels are reduced, then this is likely to interfere with intracellular cAMP generation and consequently tyrosine phosphorylation. Therefore it was of interest to observe the effects of α CH on tyrosine phosphorylation and also determine whether or not there is any correlation with inhibition of capacitation, as measured by the ability of spermatozoa to undergo the P_4 induced acrosome reaction.

The *in vitro* exposure of spermatozoa to α CH exerted a significant effect on the percentage population of acrosome reacted spermatozoa, that had been treated with PTX in combination with P_4. Interestingly, significantly high levels of physiological ARs are induced following treatment of the spermatozoa with the combination of these two reagents. ARs were also considerably reduced when compared with the same treatment in normal BWW. This indicates that α CH does not significantly affect spontaneous acrosome reactions because they occur outwith the control of the cell, irrespective of their capacitation status. As mentioned previously, spontaneous acrosome reactions may occur as a consequence of centrifugation during the protocol for analysis of acrosomal status. The fact that α CH exerts a significant deleterious effect on the PTX and P_4 stimulated acrosome reaction indicates that it interferes with cellular processes that enable the spermatozoa to undergo agonist induced acrosomal exocytosis.

Interestingly, tyrosine phosphorylation of novel proteins was induced in rat caudal spermatozoa, which had been incubated with PTX for 3 hours followed by exposure to P_4 for 30 minutes. These proteins were not phosphorylated in rat caudal spermatozoa that had been incubated in PTX, without exposure to P_4. Spermatozoa are exposed to progesterone around the oocyte *in vivo* (Benoff, 1998). These observations, together with the ability of PTX and P_4 to induce the acrosome reaction, suggest that progesterone may be important for the induction of capacitation-associated tyrosine phosphorylation, during the final stages of acquisition of fertilising ability. It has been postulated that a receptor tyrosine kinase may activate a sperm Na^+/H^+-exchanger which promotes cell alkalisation, membrane depolarisation and activation of a Ca^{2+} channel (Breitbart and Spungin, 1997). Progesterone stimulates tyrosine phosphorylation of a 94 kDa human sperm protein (Tesarik *et al.*, 1993b), which could be the 95 kDa receptor tyrosine kinase and Kopf's laboratory have also identified a 95 kDa phosphorylated protein in mouse spermatozoa with properties of hexokinase (Kalab *et al.*, 1994). This raises the possibility that one of the progesterone induced phosphorylated proteins observed in rat caudal spermatozoa, could be either a receptor tyrosine kinase, important for the changes associated with acrosomal exocytosis, or indeed a hexokinase, or AKAP.

α CH exerted a profound effect on tyrosine phosphorylation in both caput and caudal spermatozoa *in vitro*. Tyrosine phosphorylation was almost completely inhibited in both types of spermatozoa. This observation together with the evidence that α CH significantly inhibits the PTX and P_4 stimulated acrosome reaction suggests that the two processes could possibly be linked.

There was a significant dose dependent *in vivo* effect of α CH on the PTX and P_4 induced acrosome reaction. α CH did not appear to affect tyrosine phosphorylation as much as when spermatozoa were incubated directly with α CH, although there was to a certain extent, a dose dependent inhibition of phosphorylation. At the maximum dosage of 9 mg/kg/day α CH, inhibition of PTX and P_4 induced AR's, was of equal magnitude to the *in vitro* effect of α CH.

In conclusion, the ability to undergo the acrosome reaction is maturation-dependent. By raising intracellular cAMP levels in caudal, but not caput spermatozoa, the capacity to undergo the acrosome reaction in response to exposure to P_4 is increased. In addition, P_4 appears to induce the phosphorylation of a novel set of proteins in addition to those stimulated by PTX alone. α CH did appear to exert inhibitory effects on both tyrosine phosphorylation and the capacity of caudal spermatozoa to complete the acrosome reaction. However, the ability of spermatozoa to acrosome react following exposure to α CH, either *in vivo* or *in vitro*, was not completely impaired.

Chapter Nine:

General Discussion

Chapter 9. General Discussion

The studies detailed in this thesis emphasise the complex nature of rat sperm biology. The purpose of the research presented in this thesis was to investigate the cell biology of rat spermatozoa. An additional aim was to utilise the knowledge obtained to aid the development of *in vitro* functional tests for the assessment of rat sperm fertility and identify potential markers of normal epididymal maturation.

These studies investigated the impact of epididymal maturation on the signal transduction pathways regulating tyrosine phosphorylation using the laboratory rat as an animal model. The core of this work concentrated upon this process, since this signal transduction pathway is thought to be central to the attainment of a capacitated state and expression of hyperactivated motility, both of which are prerequisites for fertilisation.

Western Blot and immunocytochemical analysis demonstrated that epididymal maturation is associated with a progressive loss in phosphotyrosine expression located to the acrosomal domain. These differences in phosphotyrosine expression between caput and caudal epididymal spermatozoa appeared to reflect the normal *in vivo* situation. Interestingly, these observations are in striking contrast to a close relative of the rat, the mouse. In this species, caput spermatozoa exhibit a minimal degree of tyrosine phosphorylation, whereas cauda spermatozoa exhibit much higher levels (Visconti *et al.*, 1995a). Similarly, the rat appears to differ from other mammalian species, including the human, in that capacitation-associated changes in tyrosine phosphorylation do not occur spontaneously *in vitro*. Over a three hour incubation period in BWW medium containing all of the requisite constituents associated with the attainment of capacitation-associated phosphotyrosine expression, no changes were demonstrated in either immature caput, or mature caudal spermatozoa. However, the acrosome reaction was induced in caudal spermatozoa following incubation in BWW for a duration of three hours and progesterone for a further 30 minutes. In addition, progesterone was found to induce tyrosine phosphorylation in caudal spermatozoa. A plausible explanation is that unlike other species of spermatozoa, capacitation does not proceed spontaneously in the rat but requires external agents, such as progesterone, to promote this process. Such a hypothesis could explain why rat spermatozoa are renowned for their reluctance to capacitate *in vitro*. The reason that the acrosome reaction could be induced in a population of these untreated caudal cells may be due to the induction of capacitation and an associated increase in tyrosine phosphorylation by progesterone, in addition to its role as a natural ligand for the acrosome reaction. This

is supported by previous data obtained in other mammalian species whereby progesterone has been shown to stimulate capacitation (Emiliozzi *et al.*, 1996; Foresta *et al.*, 1992; Uhler *et al.*, 1992) and tyrosine phosphorylation (Luconi *et al.*, 1995; Martinez *et al.*, 1999; Tesarik *et al.*, 1993b) in human spermatozoa in addition to the acrosome reaction in human (Osman *et al.*, 1989), porcine (Melendrez *et al.*, 1994) and stallion (Meyers *et al.*, 1995) spermatozoa.

Epididymal maturation of rat spermatozoa was found to be associated with an acquired competence to respond to high levels of intracellular cAMP by phosphorylating tyrosine residues on the sperm tail. The inability of immature caput spermatozoa to respond to stimulators of intracellular cAMP was not due to reduced levels of adenylyl cyclase or PKA activity as measured by intracellular cAMP measurement or the detection of PKA constituents. This up-regulation in phosphotyrosine expression was correlated with the attainment of a capacitated state, as measured by the ability of mature caudal spermatozoa to undergo the progesterone-induced acrosome reaction, following incubation with stimulators of intracellular cAMP levels. Under these conditions, the population of progesterone-induced acrosome reacted caudal spermatozoa was maximal. Interestingly, it was not possible to induce the acrosome reaction in rat immature caput spermatozoa. This correlates with the current theory that most mammalian species of spermatozoa must undergo epididymal maturation in order to acquire the potential for fertilisation.

Although the various aspects of rat sperm motility have not been considered in these studies, it would seem reasonable to propose that the extensive phosphorylation of the sperm tail in caudal spermatozoa in response to increased intracellular cAMP may play a role in the attainment of hyperactivated motility. cAMP is known to be an important regulator of sperm motility, through the direct phosphorylation of specific proteins on the sperm tail (as reviewed in Lindemann and Kanous, 1989). As discussed briefly in Chapter One, changes in sperm motility patterns constitute a crucial aspect of capacitation.

Epididymal maturation also led to unique differences in the generation of reactive oxygen species (ROS) by spermatozoa obtained from the caput and caudal regions of the epididymis. Spermatozoa from both regions of the epididymis spontaneously generated equal levels of $O_2^{\cdot-}$, whereas only mature caudal spermatozoa generated significant levels of H_2O_2. In contrast, although both caput and caudal spermatozoa generated increased $O_2^{\cdot-}$ in response to NADPH, induced levels were significantly greater in the immature caput cells. The effect of NADPH on tyrosine phosphorylation was also dependent on the maturation status of the cells. Thus although NADPH stimulation increased intracellular cAMP levels in spermatozoa at all levels of

epididymal maturation, tyrosine phosphorylation of tail proteins was only observed in the mature caudal spermatozoa. The ROS-induced phosphorylation of caudal spermatozoa was also correlated with the cell's capacitation status, as the proportion of spermatozoa that responded to progesterone by undergoing the acrosome reaction was significantly greater than those cells that had not been exposed to such stimulation. These data reiterate the hypothesis that ROS have a modulating role in certain aspects of capacitation. The mechanism by which such effects are achieved could be via the stimulation of intracellular cAMP as described here and in other studies (Aitken *et al.*, 1998a; Zhang and Zheng, 1996). ROS could also exert direct actions on the sperm plasma membrane by increasing membrane fluidity as previously described (Sinha *et al.*, 1993). In addition direct effects of H_2O_2 on the activation of tyrosine kinases, or the suppression of tyrosine phosphatases would all be consistent with the existing literature (Hecht and Zick, 1992; Monteiro and Stern, 1996).

These studies have also demonstrated the requirement for bicarbonate in the incubation medium for the maintenance of $O_2^{\cdot-}$ generation and tyrosine phosphorylation. The presence of bicarbonate was important for the maintenance of ROS production, intracellular cAMP levels and tyrosine phosphorylation. Bicarbonate appears to mediate most of its effects through the maintenance of intracellular pH, as the inhibition of phosphotyrosine expression in the absence of this anion was completely overcome if the intracellular pH was restored by buffering the extracellular medium to pH 8.4. It is possible that changes in pH may switch on/off tyrosine phosphatases or kinases thus regulating the signal transduction cascade associated with this process and consequently capacitation.

Calcium was found to be a primary regulator of $O_2^{\cdot-}$ generation, intracellular cAMP and tyrosine phosphorylation in rat spermatozoa, as evidenced by the effect of exclusion of calcium from the incubation media on these processes. In addition, phosphorylation of tyrosine residues on caput sperm tails was induced under these calcium free conditions.

There are several possible models that could explain the inability of caput spermatozoa to respond to elevations in intracellular cAMP. One explanation for the enhanced inhibitory effects of calcium on phosphorylation of the flagellum in caput spermatozoa, could be the presence of calcium dependent phosphatases such as calcineurin. This could be a result of either increased calcium levels in the tail, or increased expression of such phosphatases in immature caput spermatozoa compared with mature caudal cells.

Compartmentalisation of the spermatozoon has led to the establishment of distinctly separate cytoplasmic domains within the cell, thus allowing differences in the distribution of internal antigens, ion concentration and second messengers such as cAMP. Consequently, different regulatory mechanisms may be active at discrete locations, at the same time, within the same cell. Therefore it is feasible that levels of calcium could be different in the tail than in the head region of immature caput spermatozoa, thus explaining the selective inhibition of cAMP-induced tyrosine phosphorylation in the sperm tail. Further evidence for the general concept that different ion concentrations can be compartmentalised in mammalian spermatozoa may be provided by the fact that levels of Ca^{2+} are higher in the head during the acrosome reaction and greater in the flagellum during hyperactivation (Suarez and Dai, 1995).

Similarly, differences in the ability of immature and mature epididymal spermatozoa to respond to cAMP in the sperm tail, could be due to compartmentalised differences in tyrosine kinase activity. Thus while tyrosine kinases are highly active in caput spermatozoa, as demonstrated by Western Blot analysis and immunolocalisation of numerous phosphorylated proteins in the entire acrosomal region of the sperm head, kinase activity may be selectively suppressed in the tail region. Alternatively, phosphatases may be particularly active in the tail of immature caput epididymal spermatozoa. Such phosphatase activity could involve serine/threonine phosphatases directed against the PKA-activated tyrosine kinase that is presumably responsible for the induction of tyrosine phosphorylation in caudal cells. Alternatively the disruption of PKA activated tyrosine phosphorylation in caput cells could be further downstream in the signal transduction cascade and involve the activation of tyrosine phosphatases directed against protein substrates in the sperm tail. Whatever the nature of the regulatory step that becomes modified as cells mature in the epididymis and allows them to become responsive to cAMP, it is clearly regulated by calcium. The immediate targets for calcium action in caput epididymal cells are now important to establish.

As the acrosome reaction is generally referred to as the endpoint of capacitation, the established acrosome reaction test assay was adapted to assess the capacitation and fertility status of rat spermatozoa following various treatments. Progesterone, a physiological inducer of the acrosome reaction following capacitation in mammalian spermatozoa, was also found to induce the acrosome reaction in mature caudal rat spermatozoa following a period of incubation with stimulators of intracellular cAMP. Interestingly, the reproductive toxicant, alpha chlorohydrin was found to impair the ability of rat caudal spermatozoa to undergo the progesterone-induced acrosome reaction, as well as exerting an inhibitory effect on tyrosine phosphorylation. Apart from demonstrating the potential of such an assay for use in reproductive toxicology,

these results further emphasised the relationship between the phosphorylation of tyrosine residues on specific proteins and the completion of capacitation.

The findings detailed in this thesis provide us with valuable biochemical markers to elucidate further the mechanisms of epididymal maturation by which spermatozoa acquire the potential for fertilisation as they transit the epididymis. This thesis has also identified the similarities and differences in cell biology between the rat and other species including the mouse and human, thus emphasising the importance for researchers to exercise caution in the interpretation of data from one species in relation to its impact on others.

It is also important to appreciate that these studies were mostly undertaken *in vitro* and consequently they may not be completely reflective of the *in vivo* situation. For example, although intracellular cAMP levels have been successfully manipulated in rat spermatozoa using phosphodiesterase inhibitors in association with NADPH or membrane permeant analogues of cAMP, we have no idea how [cAMP]i is normally controlled *in vivo*. It is possible that *in vivo* changes in factors such as pH and Ca^{2+} concentration are involved in switching on and off key constituents of the various signal transduction cascades involved in capacitation. Nevertheless, the use of such *in vitro* strategies, as those detailed in this thesis provide a valuable tool for elucidating the mechanisms involved in the acquisition of fertility, thus laying the groundwork for the establishment of how these specialised spermatozoa function *in vivo*.

Future Studies

Although the research detailed within this thesis has succeeded in its aim of increasing the current knowledge of rat sperm biology, as well as identifying possible means of assessing rat sperm fertility, further studies in these areas are required.

For example it is important to determine the function of tyrosine phosphorylation in rat spermatozoa, by identifying and characterising these proteins in the hope of elucidating the role they play in the acquisition of fertilising potential. Which proteins are associated with acquisition of hyperactivated motility? Are some of these proteins related to specific membrane changes that confer upon the cells the ability to undergo the acrosome reaction and membrane fusion/cell recognition events associated with fertilisation?

Another possible avenue of study would involve identification of the epididymal maturation-associated inhibitory mechanisms involved in suppressing the tyrosine phosphorylation patterns associated with sperm capacitation, while cells are in the caput epididymis. Are tyrosine kinase or tyrosine phosphatases involved? Where is such activity localised?

Although it is evident that the use of the progesterone-induced acrosome reaction has a potential use for the assessment of rat sperm fertility, much refinement of the methodology is required. These studies demonstrated high levels of spontaneous acrosome reacted caudal spermatozoa. Personal communications with other scientists suggest that this is typical of rat spermatozoa. Using the current method, significant differences between progesterone-induced and spontaneous acrosome reactions were demonstrated. However, in order to maximise the differences observed following treatments, it would be advantageous to adapt this assay for flow cytometry.

Using the same vital stain and lectin as used during our studies, this method has previously been used to measure acrosome reactions in human spermatozoa (Cooper and Yeung, 1998). The advantages associated with flow cytometry in comparison with microscopy are summarised in Table 9.1. below. Obvious advantages of flow cytometry include accuracy and improved standardisation. As evaluation of acrosome reacted spermatozoa by this method is automated, bias is eliminated and it is easier to standardise the differentiation between acrosome reacted and acrosome intact spermatozoa. In contrast, evaluation of sperm cell status by microscopy is manual and may introduce ambiguity into the assessment of acrosome status. For example, the interpretation of observations may vary among different researchers, thus raising the possibility of generating inconsistent data. Adding to the accuracy of flow cytometric methods is the fact that many thousands of cells may be analysed in seconds, and consequently the data obtained represents a truer reflection of the sperm population of

a particular sample. Due to the increased length of time it takes to evaluate spermatozoa for their acrosome status by microscopy, it is not feasible to count more than 200 sperm cells per sample. Therefore only a very small proportion of the total number of cells are analysed, limiting the accuracy of the analysis. Taken together, all of these factors indicate that the use of microscopy for routine analysis of rat sperm acrosome reaction is not a feasible option, but the adaptation of the staining methods described in this thesis for use with flow cytometry would be a suitable and advantageous alternative.

In order to determine the true value of the progesterone induced rat sperm acrosome reaction, in terms of its potential use as a reproductive toxicological tool, it is crucial that further investigations are carried out to test its efficacy in the detection of abnormalities in sperm function.

Parameters associated with accuracy of method	**Microscopy**	**Flow cytometry**
Nº of cells analysed	200	10000's +
Mode of analysis	Manual - introduces bias and variability in assessment	Automated - increases accuracy and easier to standardise evaluation
Duration of analysis	Time consuming	Time efficient

Table 9.1. Comparison of microscopy Vs flow cytometry for the analysis of acrosome reacted spermatozoa.

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Publications

Publications

Lewis, B. A. and Aitken, R. J. Impact of Epididymal Maturation on the tyrosine phosphorylation patterns exhibited by rat spermatozoa. Submitted to *Biology of Reproduction*.

Lewis, B. A. and Aitken, R. J. A redox-regulated tyrosine phosphorylation cascade in rat spermatozoa. Submitted to *Journal of Andrology*.

Lewis, B. A. and Aitken, R. J. Calcium and bicarbonate regulation of tyrosine phosphorylation in rat epididymal spermatozoa. In preparation.